DISS. ETH NO. 23312

Role of G-protein Coupled Estrogen Receptor in Mediating the Vasoprotective Actions of Estradiol

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

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2016

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Acknowledgements

During the time conducting my PhD work and writing my thesis, I received support and help from many people. At this point, it is time to thank those who made my PhD possible:

First of all, I would like to thank Prof. Raghvendra K. Dubey, for giving me the opportunity to do my PhD at the Department of Reproductive Endocrinology. Rags supported, guided and encouraged me continuously during this time. In tough times, his optimism was motivating and in less tough times we had a lot of fun.

My gratitude goes to Prof. Sabine Werner for supervising my project and for providing me helpful and valuable suggestions. For me, our meetings were very inspiring. I am thankful for the members of my PhD committee, Prof. Gebhard Schertler and Prof Christian Grimm, for their scientific advices during annual meetings.

Thanks to all current and former lab members of the Dubey lab: Lisa, Yuliya, Federica and Doris, for support, scientific discussions, daily life advices and sport activities. Special big THANKs to Lisa and Yuliya, for their patience with me in times of lots of moaning and for becoming good friends! Working in such a nice, friendly and open atmosphere was very motivating and I am very happy to know you and to have worked with you!

I also want to thank all the other people in Schlieren. Special thanks goes to the Austrian connection Johannes and Lisa for the countless rooftop-coffees in time of desperate needed breaks, to Caro for being a good fellow and to Assem and Irina, who would have thought at the interviews that we all end up together in Schlieren? And to Franzi, although not in Schlieren but Bellinzona, I shared every problem/ joy/ breakthrough with her. All of you made my PhD time a wonderful experience!

I want to thank my family and specially Berni, who understands me often better than I do, always supports me and makes me laugh so much! A life together with you is great! Finally thanks to my friends for staying and becoming my friends throughout the PhD time!

This work was supported by the Swiss National Science Foundation Grants: #31003A-138067 and #IZERO-142213/1 awarded to Prof. Raghvendra K Dubey. Salary support was provided by the SNF grant #31003A-138067 and for three months by the Hartmann Müller Stiftung (F-84003-01) #1789.

Summary

Ageing is associated with a significant increase in Cardiovascular Disease (CVD) and mortality in both men and women. Because the onset of menopause in women correlates with a significant decrease in estrogen levels, it is hypothesized that estrogen reduction importantly contributes to the increase in cardiovascular mortality in postmenopausal women. Epidemiological and clinical studies provide strong evidence that estrogen protects women treated within five years of menopause onset, against CVD. The dynamic interaction of estrogens with cells within the vessel wall, Endothelial Cells (ECs) and Smooth Muscle Cells (SMCs), contributes to its anti-vasoocclusive actions. However, the mechanisms involved remain unclear. Although Estrogen Receptors ERα and ERβ are thought to mediate the vasoprotective actions via genomic or non-genomic mechanisms, the role of GPER, a newly discovered membrane G-protein coupled Estrogen Receptor, remains unknown.

Hence, in this study we investigated the role of GPER in mediating the protective effects of estrogen on the vessel wall. Since endothelial dysfunction and abnormal growth of SMCs contribute to the vascular remodeling process leading to vascular occlusion in CVD, we investigated the role of GPER in modulating endothelial and SMC function and the underlying mechanisms mediating these effects. Using pharmacological (GPER specific agonist G1 and antagonist G15) and molecular (siRNA) approaches we assessed the role of GPER in regulating EC and SMC function. We demonstrate that GPER activation improves Human Umbilical Vein Endothelial Cell (HUVEC) function by inducing vasculogenesis, sprouting, migration and proliferation. Importantly, the effects are mediated via two key pro-vasculogenic pathways i.e. ALK1/ SMAD1/5/8 and PI3K/ Akt/ NO/ VEGF. Moreover, we show that crosstalk between these pathways is essential to facilitate vasculogenic actions of GPER. Finally, we provide evidence that GPER activation inhibits Human Coronary Artery Smooth Muscle Cell (HCASMC) proliferation and migration via downregulation of PI3K/ Akt, but not ALK1/ SMAD1/5/8, signaling.

In summary, our findings demonstrate that GPER actively mediates the growth effects of estrogen on vascular ECs and SMCs. Importantly, GPER activation induces EC function and inhibits SMC function. These differential effects of GPER on

EC and SMC growth would prevent vascular remodeling associated with vasoocclusive disorders in CVD. In conclusion, we postulate that GPER might be a novel therapeutic target for developing new strategies to treat CVD in postmenopausal women. Since in contrast to ERα and ERβ, GPER function remains unaltered in ageing vessels, GPER ligands may be superior to the currently used estrogen therapies in inducing vasoprotective actions.

Zusammenfassung

Der Alterungsprozess geht mit einem signifikanten Anstieg von kardiovaskulären Erkrankungen und einer damit verbundener Mortalität bei Männern sowie auch bei Frauen einher. Man vermutet einen Zusammenhang zwischen dem sinkenden Östrogenspiegel ab Einsetzen der Menopause und der erhöhten Sterblichkeit, aufgrund erhöhter Anfälligkeit für Herz-Kreislauf-Erkrankungen von älteren Frauen. Epidemiologische und klinische Studien stützen diese Hypothese und beweisen, dass Östrogen Frauen kardiovaskulär schützt, wenn die Therapie innerhalb der ersten fünf Jahre nach Einsetzen der Menopause begonnen wird. Die dynamische Interaktion von Östrogen mit vaskulären Gefässwandzellen (Endothelzellen und glatte Muskelzellen) trägt zu diesen schützenden, antivasookklusiven Effekten bei. Jedoch sind die involvierten Wirkmechanismen nach wie vor unbekannt. Obwohl man weiss, dass die Herz-Kreislauf-schützenden Effekte von Östrogen teils über die Kern-Rezeptoren ERα und ERβ, über genetische und nicht-genetische Mechanismen, vermittelt werden, ist die Rolle des neu entdeckten G-Protein gekoppelten Östrogenrezeptors GPER weitgehend unerforscht.

Das Ziel dieser Studie war, die Rolle von GPER als Vermittler von Östrogen und dessen schützende Effekte auf vaskuläre Blutgefässe und deren Wände zu untersuchen. Endotheliale Dysfunktion und abnormales Wachstum von glatten Muskelzellen führen zur vaskulären Okklusion und damit zu Herz-Kreislauf-Erkrankungen. Wir ermittelten, ob GPER die Funktion von vaskulären Gefässwandzellen modulieren kann und analysierten die zugrunde liegenden, molekularen Mechanismen. Mittels pharmakologischer (GPER spezifischen Agonist G1 und Antagonist G15) und molekularer (siRNA) Methoden haben wir die regulierende Wirkung von GPER auf die Funktion von Gefässwandzellen untersucht. Wir konnten zeigen, dass die Aktivierung von GPER zur verbesserten Funktion von *Human Umbilical Vein Endothelial Cells* (HUVECs) führt, indem G1 Vaskulogenese, Migration und Proliferation induziert. Entscheidend ist, dass die zugrunde liegenden Signalwege, die zu diesen Effekte führen, identifiziert werden konnten: ALK1/ SMAD1/5/8 und PI3K/ Akt/ NO/ VEGF werden von G1 aktiviert. Auch entdeckten wir, dass beide Signalwege miteinander verbunden sind, sich gegenseitig regulieren, und dieser „Crosstalk“ für die vaskulogenen Effekte von GPER essentiell ist. Schliesslich konnten wir auch zeigen, dass die Aktivierung von GPER die Proliferation und

Migration von *Human Coronary Artery Smooth Muscle Cells* (HCASMCs) inhibiert, indem G1 PI3K/ Akt, aber nicht ALK1/ SMAD1/5/8, blockiert.

Zusammenfassend zeigen unsere Ergebnisse, dass GPER aktiv die Effekte von Östrogen auf das Wachstum von vaskulären Gefässwandzellen reguliert. Hervorzuheben ist, dass GPER die Funktion von Endothelzellen induziert und die von glatten Muskelzellen inhibiert. Diese unterschiedlichen Effekte von GPER auf das Wachstum von Gefässwandzellen tragen dazu bei, vaskuläre Umbauprozesse, die zur Vasookklusion bei Herz-Kreislauf-Erkrankungen führen, zu unterbinden. Abschliessend postulieren wir, dass GPER ein Ziel für neue therapeutische Ansätze bzw. Medikamente sein könnte, um kardiovaskuläre Erkrankungen in postmenopausalen Frauen zu behandeln. Da die Funktion von GPER, im Gegensatz zu ERα und ERβ, in alternden Blutgefässen nicht beeinträchtigt ist, könnte ein spezifischer Ligand für GPER eine höhere vasoprotektive Wirkung erzeugen als herkömmliche Östrogentherapien.

Abbreviations

2ME	2-Methoxyestradiol
2OE	2-Hydroxyestradiol
4ME	4-Methoxyestradiol
4OE	4-Hydroxyestradiol
AA	Antibioticum- Antimycoticum
Akt	Proteinkinase B
ALK	Activin Receptor-like Kinase
ALK1Fc	ALK1 neutralizing antibody
ATP	Adenosine TriPhosphate
B	Batimastat, MMP-inhibitor
BCA	Bichinonic Acid
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
cAMP	3',5'-cyclic Adenosine Monophosphate
cDNA	complementary Desoxyribonucleic Acid
COMT	Catechol-O-methyltransferase
CVD	Cardiovascular Diseases
CYP 450s	Cytochrome P450 enzymes
CY	Cycloheximide, translation inhibitor
DAF-2DA	4,5-Diaminofluorescein diacetate
DDA	2′,3′-Dideoxyadenosine, an adenylate cyclase specific inhibitor
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F12
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E1	Estrone
E2	17-β Estradiol
E3	Estriol
EBM	Endothelial Basal Medium
EC	Endothelial Cells
ECFCs	Endothelial Colony Forming Cells
EGF	Endothelial Growth Factor
ELISA	Enzyme-linked Immunoassay

ER	Estrogen Receptor
ERE	Estrogen Response Element
FCS	Fetal Calf Serum
G1	GPER specific agonist
G15	GPER specific antagonist
GDP	Guanosine DiPhosphate
GPCR	G-protein coupled Receptor
GPER	G-protein coupled Estrogen Receptor
GRK	G-protein coupled Receptor Kinase
GTP	Guanosine TriPhosphate
HBSS	Hank`s Buffered Salt Solution
HCASMCs	Human Coronary Artery Smooth Muscle Cells
HCl	Hydrochloric acid
HERS	Heart and Estrogen/ Progestin Replacement Study
HHT	Hereditary Hemorrhagic Telangiectasia
HRT	Hormone Replacement Therapy
HSD	Hydroxysteroid Dehydrogenase
HUVECs	Human Umbilical Vein Endothelial Cells
ICI	ICI 182-780, fulvestrant, ER unspecific antagonist
IL	Interleukin
L-NAME	eNOS inhibitor
L-NMMA	eNOS inhibitor
LSGS	Low Serum Growth Supplements
LY	LY294002, PI3K-inhibitor
MEK	MAPK, mitogen activated protein kinase
Mg^{2+}	Magnesium
mm-IBMX	8-Methoxymethyl-3-isobutyl-1-methylxanthine
MMP	Matrix Metallo Proteinase
MPP	Methyl-Piperidino-Pyrazole, ERα unspecific antagonist
mRNA	messenger Ribonucleic Acid
NO	Nitric Oxide
PAH	Pulmonary Arterial Hypertension
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PDGF	Platelet Derived Growth Factor
PI3K	Phosphoinositide 3 Kinase

PDK1	Phosphoinositide-dependent kinase 1
PKA	Protein kinase A
PKI	PKI (5-24), a PKA-specific inhibitor
PPT	Propy Pyrazole Triol, ERα specific agonist
PTX	Pertussis Toxin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-PCR	Real Time quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SERDs	Selective Estrogen Receptor Downregulators
SERMs	Selective Estrogen Receptor Modulators
siRNA	small interfering RNA
SJN	SJN2511, an ALK5 inhibitor
SMCs	Smooth Muscle Cells
SMGS	Smooth Muscle Growth Supplement
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor
VEGFAb	neutralizing antibody for VEGF
VSMCs	Vascular Smooth Muscle Cells
WHI	Women`s Health Initiative

1 Introduction

1.1 Estrogens and their Physiology

Estrogens are the primary female sex hormones. The naturally generated estrogens in women are estrone (E1), 17-β estradiol (E2) and estriol (E3). Although E2, E3 and E1 are present in women of all age, in adult non-pregnant females E2 is predominant, while E1 are high in menopausal females and E3 levels dominate in pregnant females [1].

1.1.1 Estrogen`s Biological Effects

Estrogens regulate growth and function of female reproductive organs and promote development of female secondary sex characteristics. Furthermore, they mediate the menstrual cycle and are critical for the fertilization processes and maintenance of pregnancy. Apart from an essential role in reproductive organs, estrogens also play a critical role in regulating physiology and biology of many other tissues. For example in the brain estrogen has neuroprotective effects and protects against Alzheimer`s disease; it improves cardiovascular function and protects against Cardiovascular Diseases (CVDs) (described in detail in section 1.5); estrogen ameliorates metabolism of cholesterol and lipoproteins in the liver; estrogen increases bone density and therefore protects against osteoporosis; in the gastrointestine estrogens reduce the risk of colon cancer; and estrogen improves skin aging [2, 3]. Apart from the beneficial effects of estrogen listed above; the pro-mitogenic characteristics of estrogen in breast and uterine can also induce uterus and breast cancer [4]. Although estrogens are primary female sex hormones, they also occur in males and influence the male reproductive system and fertility [2].

1.1.2 Estrogen Synthesis

Estrogens are mainly produced within female ovaries [5], but other tissues can also synthesize estrogens. For example, estrogens are also produced in brain, liver, adipose tissue, skin, muscle, breast, vaginal mucosa and endometrium.

Estrogens and other steroid hormones, like progestins and androgens, are synthesized from cholesterol. The synthesis starts by uptake of cholesterol into steroidogenic cells, in which specific cytochrome P450 enzymes (CYP 450s) convert it to pregnenolone. Via two different mechanisms pregnenolone is further processed

enzymatically to testosterone: either by the actions of 3-β hydroxysteroid dehydrogenase (HSD) and 17-β HSD or hydroxylation of 17-α position by 17-α-hydroxylase to 17-α-hydroxypregnenolone, which is further converted by 17,20 lyase to dehydroepiandosterone and subsequently by 17-β HSD to testosterone. Finally, aromatase converts testosterone to 17-β estradiol (as depicted in Figure 1).

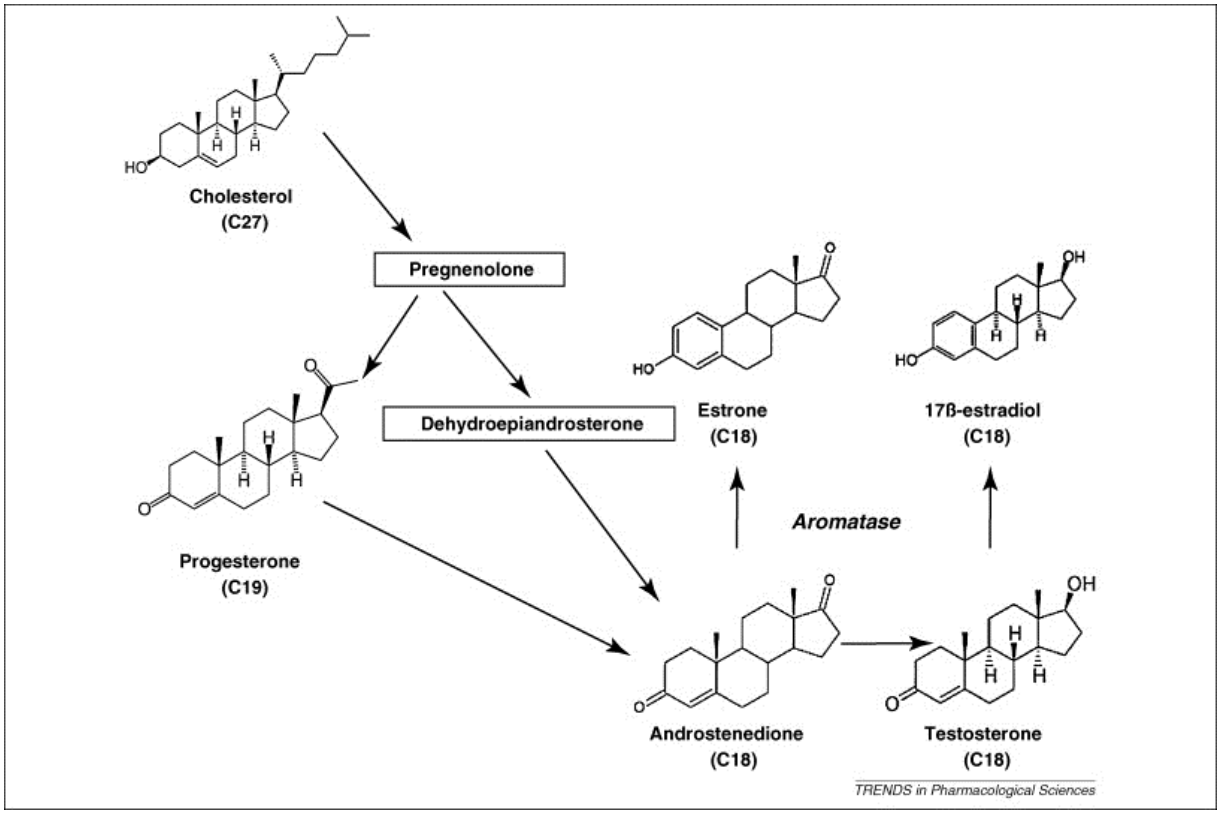

Figure 1: Estrogen Synthesis [6]. **C**: Carbon molecules.

Following synthesis estradiol is released into the circulation. It is present either in free form (2-3% of total amount) or bound to albumin or sex-hormone binding globulin. Circulating concentrations of estrogens range from 0.36 nmol/L in the follicular phase to 2.2 nmol/L in ovulation phase. The circulating levels of estrogens peak during pregnancy and reach up to 73 nmol/L and drop after menopause to 0.02 nmol/L, which is comparable to estrogen concentrations in men [7].

1.1.3 Estrogen Metabolism

The elimination of estrogen is facilitated by its conversion to water-soluble metabolites, which are excreted via urine or feces. Estrogen is conjugated either to sulfates, to glucuronides or to fatty acids and subsequently degraded. Moreover, estrogen can also be metabolized into bioactive products: estrogen is converted into oxidative metabolites as described in Figure 2, e.g. 2-hydroxyestradiol (2OE) and 4-

hydroxyestradiol (4OE) by CYP450, followed by methylation to 2-methoxyestradiol (2ME) and 4-methoxyestradiol (4ME) by catechol-O-methyltransferase (COMT).

Mainly, this metabolism occurs in the liver, but CYP450 and COMT are ubiquitous enzymes, which are also present in other tissues, like vasculature, kidneys, gastrointestinal tract, spleen, brain and pancreas [8, 9]. Although the majority of estrogen metabolites are excreted, some of them have significant growth regulatory effects [8]. Consequently, 2ME has been reported to reduce neovascularization, tumor growth [10], neointima formation and proliferation of Smooth Muscle Cells (SMCs) [11].

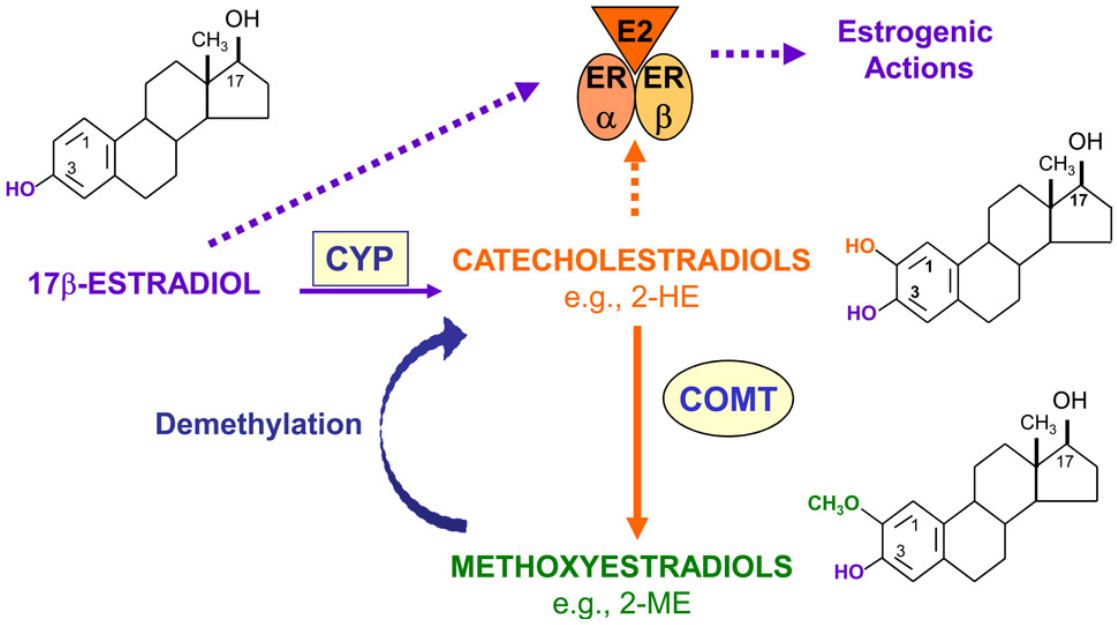

Figure 2: Estrogen Metabolism, depicted and modified from Dubey et.al. [12]

1.2 Estrogen Receptors

1.2.1 Estrogen Receptor α and Estrogen Receptor β

Estrogenic effects are mainly mediated via Estrogen Receptors (ERs). The classical ERs are ERα, which was discovered in 1960 and cloned for the first time in 1986 [13], and ERβ, which was discovered in 1996 [14]. The genes of ERα and ERβ are located on chromosome 4 and 6 and encode for proteins containing 595 and 530 amino acids, respectively [15]. 95% of ERα and ERβ are located inside the cell nucleus, while 5% are present in the cytoplasm [16].

Like all members of classical nuclear receptors, ERα and ERβ inherit 6 conserved domains A-F, as depicted in Figure 3. A/B represent the N-terminal domain, which

contains the activating transcription function 1 (AF1), being responsible for ligand independent transcription activation. The highly conserved domain C is responsible for DNA binding via two zinc finger structures. D is the hinge region, responsible for recruiting and binding of co-modulators, while E and F contain the ligand-binding domain, which, together with activating transcription function 2 (AF2), is the ligand-dependent activator of transcription [17]. Apart from the DNA-binding domain C, which is 95% similar, the homology in ERα and ERβ is low. However, the binding affinity of the endogenous ligand E2 to both ERα and ERβ is Kd=0.6 nmol/L [14].

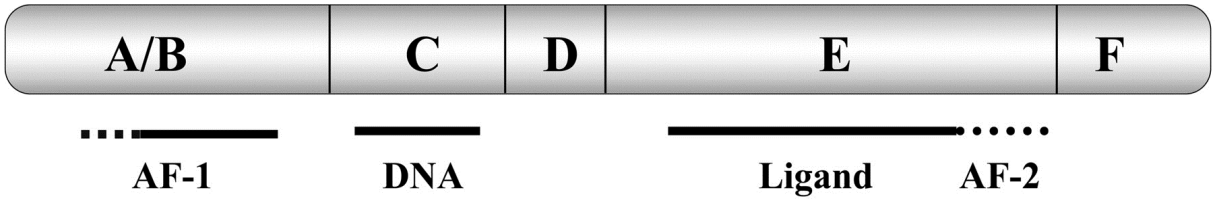

Figure 3: Structure of ERα and ERβ [17]

The tissue distribution of ERα and ERβ is diverse: while ERα is prevalent in the endometrium, breast cells and in the ovary, ERβ is mainly expressed in the kidney, the intestinal mucosa, bones, the lung and the prostate [2]. The effects of ER-ligand E2 depend on the regulation of ER, and E2 has been shown to induce ER expression in breast cancer cells [18, 19], retinal pigment epithelium [20] and osteoclasts [21].

The investigation of differential effects and roles of ERα and ERβ is facilitated by commercially available agonists and antagonists: ICI 182-780 is an ER unspecific antagonist, MPP is an ERα specific antagonist, PPT is an ERα specific agonist, PHTPP is an ERβ specific antagonist and DPN is an ERβ specific agonist.

1.2.2 GPER, the G-protein Coupled Estrogen Receptor

In addition to the classical Estrogen Receptors α and β, a third membrane bound Estrogen Receptor has recently been identified.

Subsequently, between 1996 and 1998, several laboratories independently discovered and cloned an orphan receptor [22-27], the ligand for which was unknown and named it GPR30 because it was thought to belong to the family of the classical seven-transmembrane G-protein coupled receptors.

As depicted in Figure 4, the structure of classical G-protein coupled receptors is highly conserved and can be divided into three different parts. The extracellular

region consists of the N-Terminus and three extracellular loops ECL1-ECL3, the transmembrane region of seven α helices TM1-TM7, and the intracellular region of three intracellular loops ICL1-ICL3, an intracellular amphipathic helix H8 and the C terminus. Access of the ligand is modulated by the extracellular region, while the transmembrane region facilitates ligand binding and further transduces the information by changing the conformation of the intracellular region, which consists of the C-Terminus and interacts with cytosolic signaling proteins [28]. Those intracellularly bound signaling proteins are the G-proteins Gα and Gβγ, G protein-coupled receptor kinases (GRKs) and β-arrestins [29, 30].

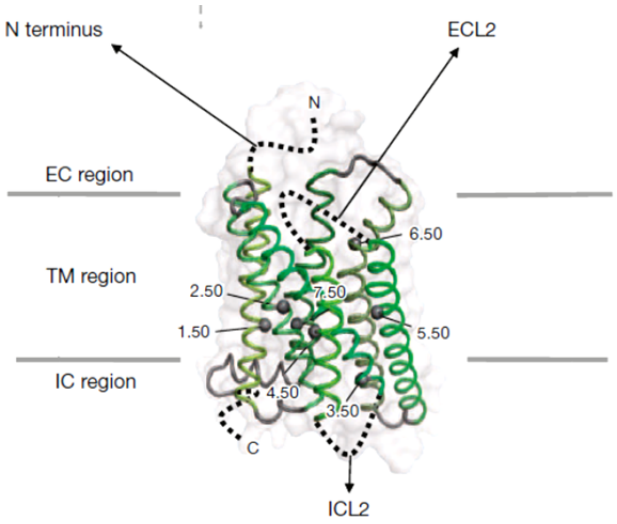

Figure 4: Structure of the classical GPCRS, depicted and modified from Venkatakrishnan et al. [28]

The model of GPCR signaling is depicted in Figure 5 and as follows: Upon ligand binding the GPCR conformation is switched to an active state, which leads to exchange of GDP to GTP, associated with the Gα subunit. The Gβγ dimer and Gα subunit dissociate from the GPCR, thus further binding and activating effectors, which themselves induce different kinds of second messengers. Gα possesses an intrinsic GTPase activity, converting GTP to GDP, thereby inactivating the G protein signaling cascade [31].

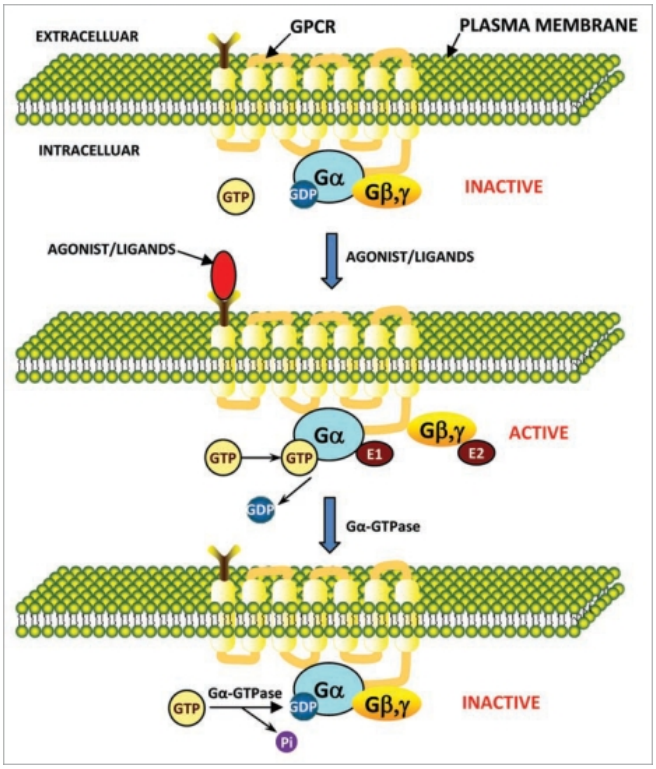

Figure 5: Classical Signal Transduction Model of GPCRs [31]

Figure 6a depicts the action of other intracellular GPCR proteins. GRKs mediate the phosphorylation of GPCR`s serine residues at the C-terminus [32], leading to the recruitment and binding of β-arrestins. These β-arrestins are scaffolding proteins, which mediate the internalization of GPCR via Clathrin Coated Pits (CCPs). This internalization process is well characterized and believed to be responsible for desensitization and impairment of GPCR signaling by recycling or transporting the GPCR to the proteasome [33]. However, recent studies suggest that β-arrestins also transduce signals to multiple effector pathways [34]. Depending on the ligand bound to GPCR, β-arrestin-mediated signaling or “normal” G-protein mediated signaling will be activated. This differential effect is termed as functional selectivity or biased signaling (Figure 6b) [35].

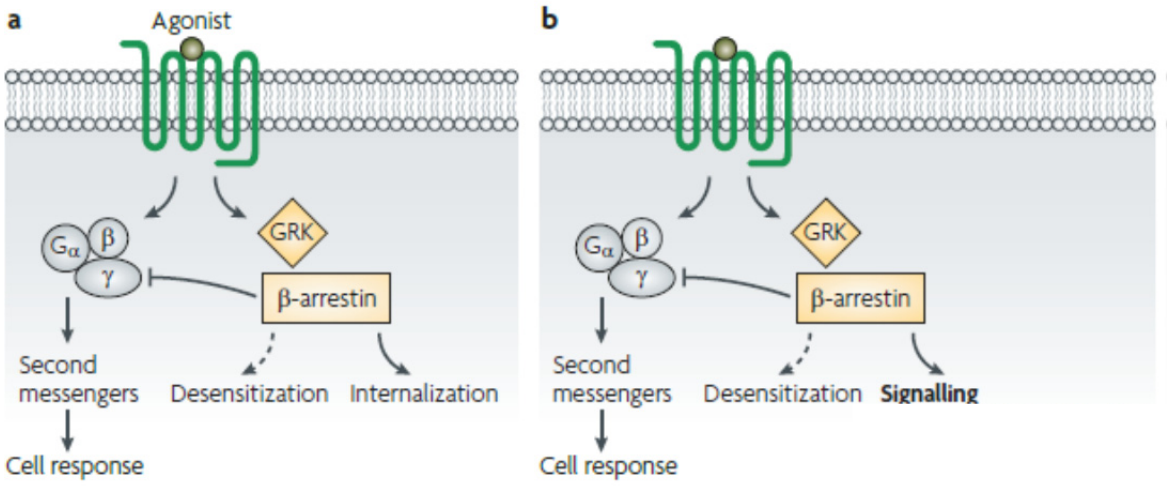

Figure 6: GRK- arresting Signaling of GPCR; **Panel a:** classical β-arrestin initiated internalization of GPCR, **Panel b:** β-arrestin-mediated signaling; depicted and modified from Rajagopal et al. [34]

Depending on the subtype of G proteins, different signaling cascades are elicited. Activation of Gα proteins (Gα12/13, Gαs, Gαi/o or Gαq/11) regulates calcium, potassium channels, adenylyl cyclase, phospholipase C, phospholipase D and protein kinases. Additionally, the Gβγ dimer activates effectors like K^+ channels, phospholipase C-β, phospholipase A2 and PI3K (Figure 7) [31, 36, 37].

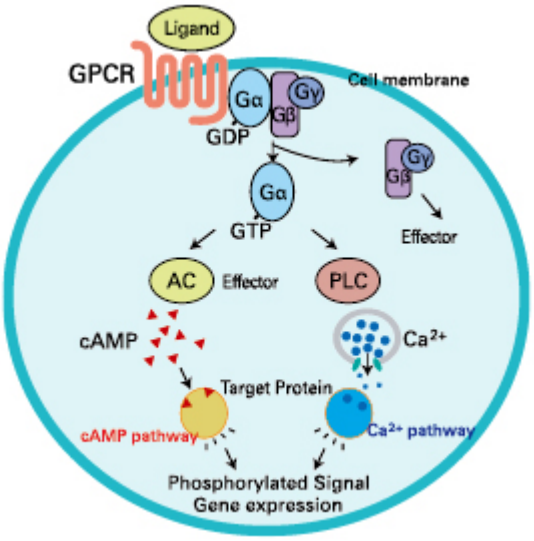

Figure 7: Representative Scheme of Signaling Cascades downstream of G protein subtypes [https://www.mblintl.com/research/gpcr.aspx, 13.11.2015 11:58 a.m]

In 2000 Filardo et al. discovered that GPR30 expression is necessary for estrogen-mediated stimulation of ERK1/2 [38] and subsequently in 2002 reported its role in cAMP generation [39]. Estrogen binding to GPR30 was demonstrated in 2005 by Revankar et al. and Thomas et.al [40, 41], and estrogen was identified as the

endogenous ligand, leading to the change of nomenclature from GPR30 to G-protein coupled Estrogen Receptor (GPER) in 2007 [42]. GPER is suggested to belong to the class A rhodopsin like-GPCRs, which occlude their ligand binding pocket sites with the extracellular region, presuming that hydrophobic ligands like estrogens, are able to bind [43, 44]. The GPER-selective ligand/ agonist G1 was identified in 2006 [45] and the GPER selective antagonist G15 discovered in 2009 [46], thereby facilitating to study the impact of GPER in mediating non-nuclear estrogenic effects. Recently, the number of studies addressing the possible cellular and physiological functions of GPER has increased exponentially.

The gene for GPER is located on chromosome 7, and the protein consists of 375 amino acids [47]. While the structure of GPER is still unknown, research suggests that GPER is linked to the G protein subtypes Gαs [41], inducing adenylyl cyclase activation and cAMP generation [39], and Gαi/o [42], which opposes Gαs as it inhibits adenylyl cyclase activation [48]. The cellular location of GPER remains unclear, while some studies state that GPER is a membrane receptor [41, 49], others suggest intracellular localization, mainly within the Endoplasmic Reticulum [40, 50, 51].

The expression and functions of GPER have been described in the reproductive, endocrine, urinary, nervous, immune, musculoskeletal and cardiovascular systems. Therefore, in combination with the estrogenic actions mediated by the classical ERs, GPER mediated actions add to the complexity of estrogen triggered signaling mechanisms and their biological and pathophysiological relevance.

Because more than 50% of drugs worldwide are designed to target GPCRs and treat cardiovascular, metabolic, neurodegenerative, psychiatric, and oncologic diseases [52], it is important to fully elucidate the role of GPER, which is proposed to be clinically relevant in cardiovascular diseases. Since coronary artery disease is a leading cause of mortality in women, and timely estrogen therapy is protective, GPER might be an important target for developing new drugs against CVDs.

1.3 Estrogen`s Genomic and Non-Genomic Actions

As depicted in Figure 8, the genomic and non-genomic actions of estrogens are elicited via ER receptors.

1.3.1 **Genomic Actions**

ERs are associated with chaperones to stabilize ER structure and mask C, the DNA binding domain. Classically, E2 is permeable and diffuses into the cell, binding to intracellular ERs and thereby triggers the disassociation from the chaperones and the homo- or heterodimerization of ERα and ERβ. This E2-ER complex translocates into nucleus and binds to a specific palindromic, cis-acting DNA sequence called Estrogen Responsive Element (ERE), which is positioned within the regulatory regions of target genes. Subsequently, the target-gene expression is either induced or repressed and regulated by coactivators or corepressors of ERs (Figure 8 A) [53].

There are also studies reporting that the E2-ER complex induces the expression of genes missing the ERE sequence. ERs interact with transcription factors Jun, Fos and Sp1 and thereby activate the respective regulatory elements [54]. Expression of Epidermal Growth Factor (EGF), EGF receptor and cyclin D1 is also upregulated by E2, although the promoter regions of their genes do not include ERE sequences (Figure 8 B) [55].

ERs can also be activated in a ligand-independent fashion. Cross-talk with extracellular growth factors like IGF-1 and EGF also stimulate the expression of ER target genes. Kato et al. suggested that phosphorylation of ERs contributes to this ligand-independent activation (Figure 8 C) [56].

1.3.2 **Non-Genomic Actions**

Estrogen`s non-genomic effects are rapid actions, which do not involve direct ER-mediated gene transcription. Estrogen-induced effects have been reported to occur within seconds to minutes, in contrast to the transcriptional mechanisms, which take hours to days, following stimulation. These rapid non-genomic effects have been reported to be initiated at the membrane level and potentially involve ERα [57] and GPER [41, 49], which have been identified within the plasma membrane of cells. Rapid effects of estradiol include regulation of mitogen-activated kinases ERK1 and ERK2 [58]; stimulation of eNOS and NO synthesis [59]; activation of cAMP formation, PKA and PKC; and increase in intracellular Ca^{2+} [60]. Those rapid effects also mediate different cellular functions and might eventually also lead to gene expression (Figure 8 D).

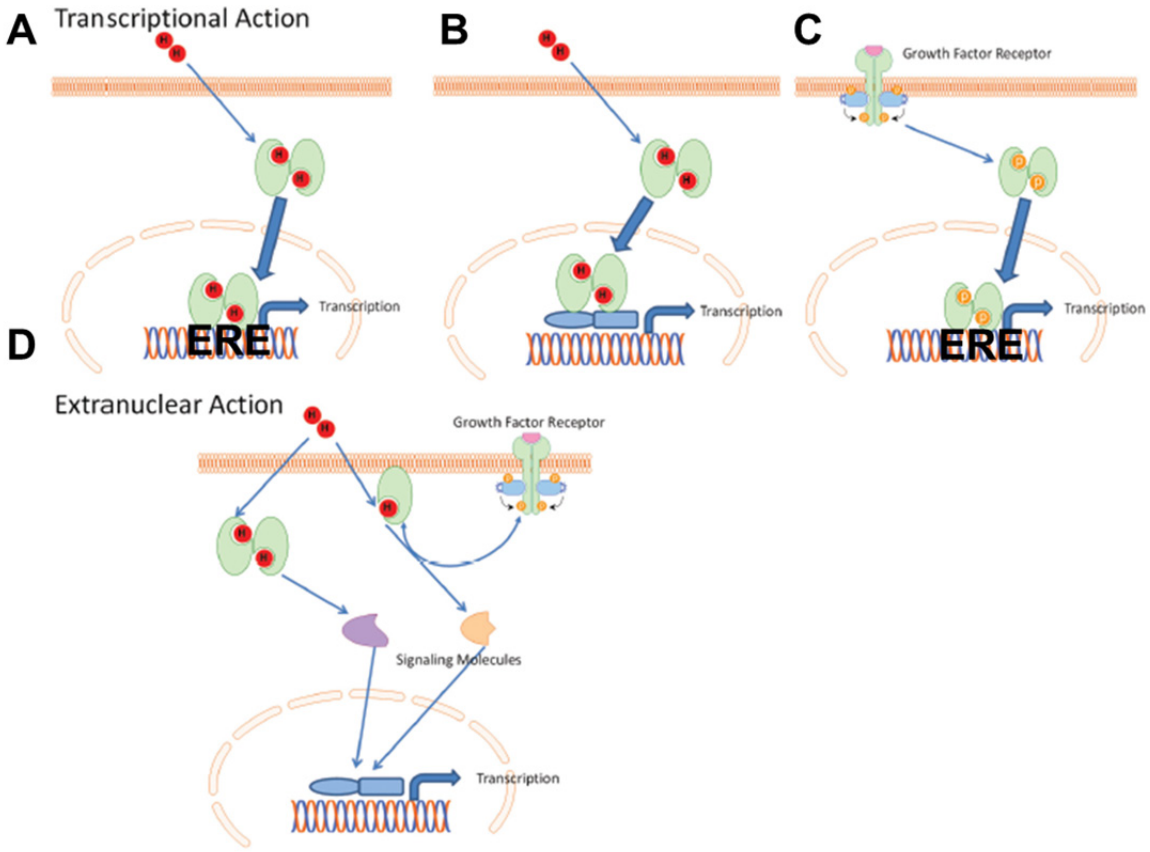

Figure 8: Genomic and Non-Genomic Actions of Estrogen Signaling. **Panel A:** Classical Genomic Action of Estrogen Signaling. **Panel B:** ERE-independent Genomic Action of Estrogen Signaling. **Panel C:** Ligand-independent Genomic Action of Estrogen Signaling. **Panel D:** Non Genomic Action of Estrogen Signaling. Depicted and modified from Kampas et al. [61].

1.4 Cardiovascular Diseases

1.4.1 Atherosclerosis and Cardiovascular Diseases

CVDs are the major cause of death worldwide [62]. The onset for the progress of CVDs is atherosclerosis, which has been correlated with the disruption of an anatomically and functionally intact endothelium [63] and abnormal differentiation and growth of Vascular SMCs (VSMCs) [64]. The steps leading to atherosclerosis are depicted and described in Figure 9. Briefly, damaged Endothelial Cells (ECs) secrete cytokines and express adhesion molecules, thereby attracting and binding monocytes. These monocytes translocate into the subendothelium and differentiate into macrophages, which digest Low Density Lipidproteins (LDL), resulting in their transformation to foam cells. Platelets are also present at the lesion site and secrete growth factors such as Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor β (TGFβ). These factors stimulate the intimal Smooth Muscle Cells (SMCs) to migrate into the lesion site, proliferate and produce connective tissue

matrix. Moreover, SMCs can take up LDL and differentiate into foam cells, subsequently all these processes lead to neointima formation and thickening of the vessel wall. The resulting plaques are unstable and can rupture. Importantly, the thrombus formed can obstruct vessels, leading to ischemic stroke or myocardial infarction and eventually to death [65].

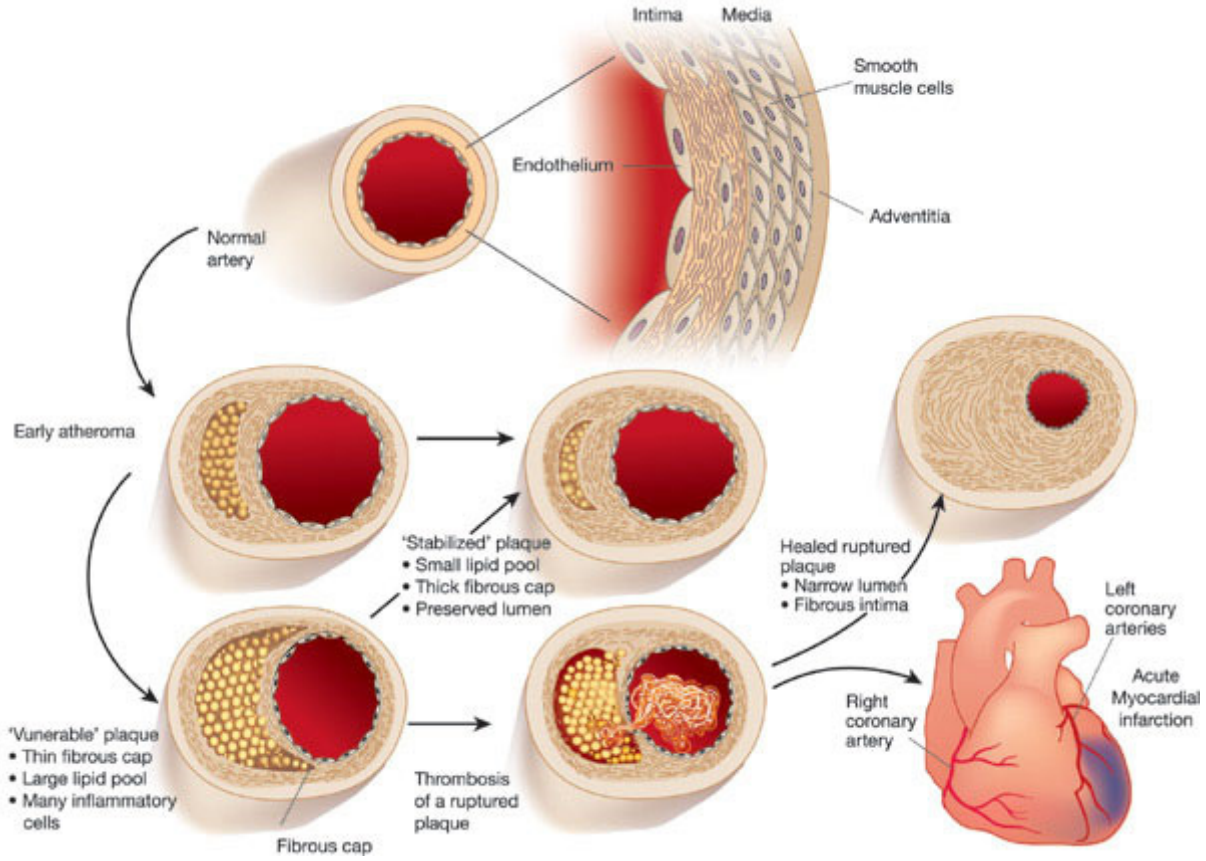

Figure 9: Schematic Representation of Steps, leading to the Onset and Progression of Atherosclerosis and subsequently to CVDs [65].

1.4.2 Role of Vascular Cells in CVDs

Endothelial Cells

As described above, endothelial damage or dysfunction initiates the onset of atherosclerosis, therefore accelerated recovery or substitution of damaged ECs may improve endothelial function and be essential to prevent the progression of atherosclerosis [63]. The hallmarks of endothelial function are viability, proliferation, and migration and the ability to form new vessels via vasculogenesis and/ or angiogenesis, to supply the tissue with oxygen and nutrients [66]. Angiogenesis is a process in which new vessels are generated from the existing ones and takes place during embryogenesis and in the adults. The term vasculogenesis describes the

assembly of angioblasts, which differentiate into endothelial cells and build vessels *de novo* [67-71]. This process was considered to only occur during embryogenesis, but in 1997 Asahara et al. discovered that Endothelial Colony Forming Cells (ECFCs), circulating in peripheral blood, participated in vasculogenic processes [72]. The common cell model for vasculogenesis assays and hence to assess endothelial function are Human Umbilical Vein Endothelial Cells (HUVECs) [73].

Smooth Muscle Cells

Another key role in the progress of atherosclerosis is the abnormal proliferation, migration and differentiation of SMCs, leading to neointimal thickening [64] and plaque formation. These processes are stimulated by cytokines and growth factors, released by damaged ECs [65]. A healthy endothelium has an inhibitory effect on SMC growth, hence enhanced recovery of endothelial function can inhibit SMC proliferation and migration and this may prevent the progression of atherosclerosis and CVDs. The controlled and balanced crosstalk between ECs and SMCs is essential for the maintenance of a functional blood vessel [74].

1.5 Estrogen`s Effects on the Cardiovascular System

The risk factors, promoting atherosclerosis and subsequently CVDs, are age, hypertension, diabetes, cholesterol, obesity, lifestyle and male gender [75]. As depicted in Figure 10, epidemiological studies have demonstrated that gender difference plays a role in the prevalence of CVDs. Moreover, elderly, postmenopausal women show a higher incidence of CVDs compared to younger, premenopausal women. These findings suggest that declining levels of endogenous estrogens upon onset of menopause (around the age of 55) are associated with increased risk of obtaining CVDs [76]. This notion is supported by the findings of Seroz et al., who showed an increased risk of CVDs in premenopausal women, undergoing surgically-induced menopause [77]. The protective effects of endogenous estrogens and Hormone Replacement Therapy (HRT) against the progression of CVDs are also supported by multiple epidemiological studies and small clinical trials [78-80]. However, no beneficial effects of estrogens on CVDs were obtained within the Women`s Health Initiative (WHI) trial and the Heart and Estrogen/ Progestin Replacement study (HERS) [79, 81, 82]. Although the reasons for the discordant findings are unclear, re-evaluation of the data from these studies demonstrated that

estrogen replacement therapy is indeed effective and protective in younger healthy women, in contrast to older study participants with established cardiovascular pathology [81, 82]. Studies in primates confirm this observation, reporting that estrogen replacement, started at the time of ovariectomy, reduced lesion formation by 70%, but showed no effects when the treatment was delayed by two years [83]. Taken together, these findings suggest that timing of HRT initiation after menopause is critical for estrogen-induced vasoprotection.

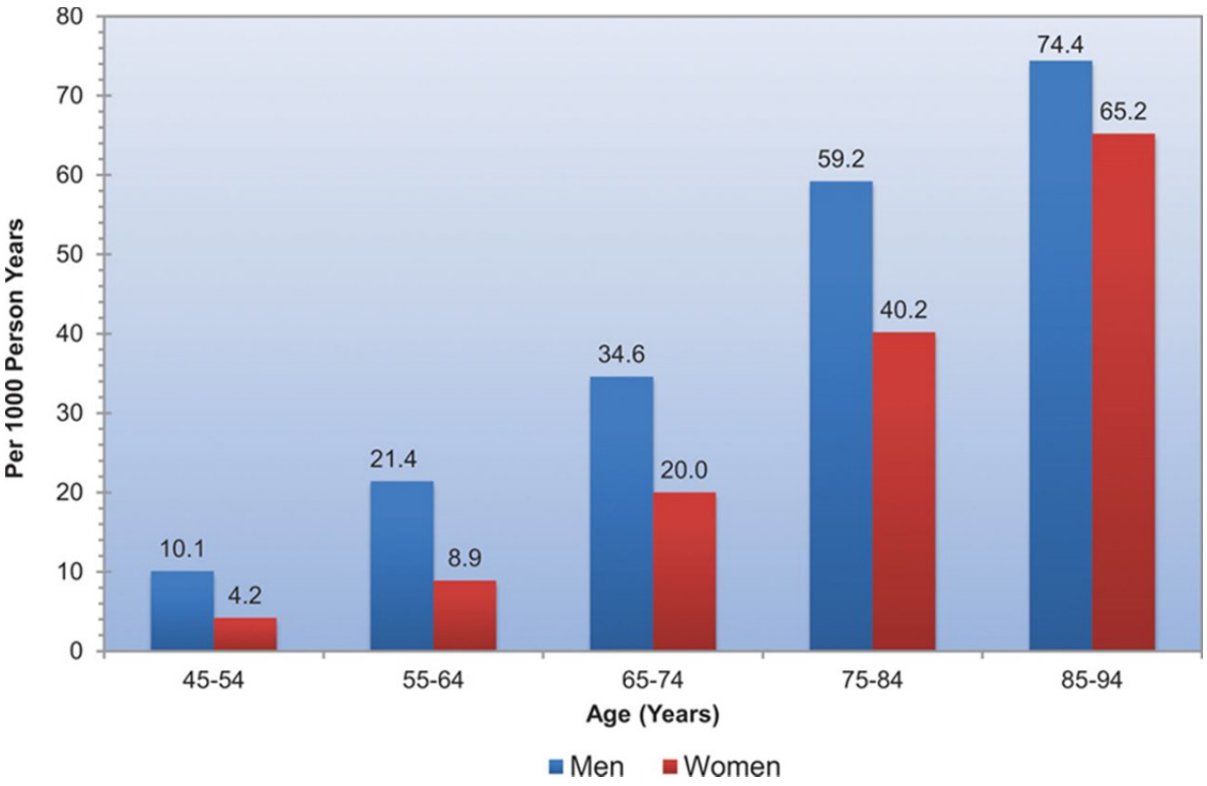

Figure 10: Incidence of CVDs by Age and Sex (Framingham Heart Study, 1980–2003) [84]

1.5.1 Estrogen`s Effects on Vascular Cells

Several *in vivo* studies support beneficial effects of HRT and demonstrate that E2 prevents pathological vascular remodeling processes like neointimal thickening, cholesterol induced atherosclerosis, balloon injury-induced neointima formation, and allograft-induced dysplasia [85-92]. Estrogen signaling elicits various pleiotropic effects, which affect the cardiovascular system by directly modulating vascular cell function or indirectly by inducing systemic effects [93].

Direct estrogenic effects on vascular cells include the stimulation of proliferation and migration of ECs *in vitro* [94] and promotion/ regeneration of ECs after arterial injury [95, 96]. Moreover, E2 might contribute to the repair of the endothelium via

mobilization of Endothelial Colony Forming Cells (ECFCs), inflammatory immune cells and platelets [97, 98]. Additionally E2 stimulates angiogenesis, both *in vitro* and *in vivo* [99-101] by inducing the most important pro-angiogenic factors: Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF) and Nitric Oxide (NO) [99].

These beneficial estrogenic actions on the endothelium might play the key role in inducing cardiovascular protection [102], however a controlled and balanced coordination between ECs and SMCs is also essential [74]. Acceleration of the endothelial integrity by estrogen can additionally contribute to its vasoprotective effects by inhibiting SMC proliferation. These anti-mitogenic effects of estrogens on SMCs are elicited via upregulation of second messengers like NO, cGMP [103], cAMP [104] and reducing the levels CA^{2+} [105]. Another protective effect of estrogen is that it blocks migration of SMCs [105, 106], which contributes to myointimal hyperplasia following injury [87, 88].

Additionally, estrogen treatment induces endothelial NO and prostaglandin synthesis, which is also responsible for the vasodilatatory and growth inhibitory effects of E2. Furthermore, E2 inhibits the production of vasoconstrictors angiotensin II, endothelin-1 and catecholamine and thereby stimulates the vasodilation of vessels even more [78].

1.6 GPER and the Cardiovascular System

An explanation for the controversial outcomes of the different trials on estrogen`s protective effects on CVDs may in part be the complexity of vascular estrogen signaling, which involves at least three different estrogen receptors: ERα, ERβ and GPER. Indeed all three receptors are expressed in the cells of the vascular system in both humans and animals of both sexes [25, 107-114]. Taken together, these findings suggest that in addition to ERα and ERβ, GPER may also play an important physiological role in regulating vascular and myocardial function.

GPER is a transmembrane G-protein coupled receptor that mediates non-genomic, rapid estrogen signaling [38, 40, 41]. The first potential relevance of GPER in cardiovascular function was suggested by Takada et al, who cloned cDNA of GPER from RNA of human ECs, which were exposed to fluid shear stress [25]. The identification of the GPER-selective ligand/ agonist G1 in 2006 [45], the GPER

selective antagonist G15 in 2009 [46], and the generation of genetically modified animals has facilitated research to define the functional role of GPER within the cardiovascular system [115]. Vasodilatory properties of GPER were suggested in studies with mammary and porcine coronary arteries, rat aorta and mesenteric arteries and rat and murine carotid arteries, where G1 induced acute dilation [108, 113, 114, 116, 117]. This G1-dependent effect on vasodilation was even more prominent than that of E2, indicating a complex crosstalk between GPER, ERα and ERβ [113]. The importance of GPER in controlling the vasomotor tone was underlined when the acute G1-induced vasodilation was lost in GPER knockout mice [113] or upon G15 pre-treatment [116, 118]. Moreover, the GPER-deficient mice were reported to develop hypertension [119]; and intravenous injection of G1 reduced mean arterial blood pressure [113] and lowered blood pressure in normotensive [114] and hypertensive rats [108]. Importantly, G1 treatment reduced the expression of angiotensin II type I receptor and angiotensin-converting enzyme in mRen2.Lewis rats, suggesting that G1 reduces high blood pressure in part by inhibiting vascular angiotensin II signaling [114]. Finally, emphasizing the importance of GPER for vascular tone, recently a hypofunctional genetic variant of GPER has been found to be associated with elevated blood pressure in women [120].

In summary, these studies provide evidence for an important role of GPER in regulating the vascular tone in response to estrogen, but the molecular mechanisms involved remain unclear and need to be further investigated.

Recent findings suggest that GPER-mediated vasodilation is in part endothelium-dependent and involves endothelium-derived NO, as the NOS inhibitor L-NAME could inhibit G1-induced relaxation in rat aorta, carotid, mesenteric and porcine coronary arteries [108, 116, 117]. Furthermore, GPER activation regulates potassium efflux, possibly via ERK and PI3K/ Akt signaling, and therefore induces endothelium-independent vasodilation [121]. The vascular tone might also be reduced by GPER`s ability to scavenge Reactive Oxygen Species (ROS), because G1 inhibits ROS production [108]. An increase in ROS was shown to damage endothelial function, by attenuating NO bioavailability. Moreover, via ROS, angiotensin II type I receptor signals induces vasoconstriction [122]. In the presence of impaired endothelial function, due to hypertension or atherosclerosis, an additional beneficial mechanism of GPER might be the regulation of vascular tone by inhibiting ROS.

Taken together, estrogen induced vasodilation is mediated by ERα, ERβ and GPER, and largely involves endothelium/ NO-dependent vasodilation. In addition to its vasodilatory effects, GPER also regulates VSMC proliferation and apoptosis [110, 113]. The fact that E2 inhibits the proliferation of VSMCs in ERα/ ERβ double knockout mice [123] suggests that the anti-mitogenic effects may have been GPER mediated and implies a protective role of GPER for atherosclerosis. This possible atheroprotective effect of GPER is underlined by a study with GPER-deficient mice, which exhibited increased adipogenesis, inflammatory activation and circulating lipid levels [124].

Cultured cardiomyocytes [125], murine [119] and human [126] myocardium express GPER. Contraction in murine cardiomyocytes as well as inhibition of calcium flux is mediated by E2, but independent of ERα and ERβ [127]. More recently, left-ventricular dysfunction was observed in male, but not female GPER-deficient mice [128]. Furthermore, G1 treatment improved diastolic dysfunction, cardiac hypertrophy and decreased myocyte size [129]. Additionally, increased expression of GPER was mediated by Hypoxia-Inducible Factor HIF-1α in a murine cardiomyocyte-like cell line, suggesting an involvement of estrogen signaling via GPER in ischemic heart disease [125].

Several groups, using cardiac ischemia/ reperfusion injury models, described additional cardioprotective effects, which were GPER-dependent. Treatment with G1 attenuated infarct size and post-ischemic contractile dysfunction in rat hearts [109, 111, 126, 130], independent of sex, but most likely mediated via the PI3K/ Akt signaling pathway [109]. Likewise, reduction of infarct size and amelioration of heart function in male mice was reported upon G1 treatment, which could be inhibited by ERK signaling blockers [107]. Furthermore, GPER mediated ERK activation was responsible for vasodilatory effects and induced eNOS phosphorylation [111], leading to an increase in NO bioavailability. Importantly, decreased levels of proinflammatory cytokines, including Tumor Necrosis Factor (TNF-α), Interleukin (IL)-1β and IL-6, have been correlated to improved heart function, upon G1 treatment [130].

In summary, activating GPER ameliorates functional recovery and infarct size after myocardial ischemia, which is in line with GPER`s dilatory effects. However, more research is needed to fully understand the molecular mechanism by which GPER

mediates its protective actions. In addition to the protective effects on the heart, GPER might be also beneficial after ischemic stroke [131] and liver injury [132].

Recent studies reported that several beneficial effects of estrogens on vascular and myocardial function are mediated by GPER. Estrogen signaling and cardiovascular response following estrogen treatment have become increasingly complex, due to the identification of three different estrogen receptors - ERα, ERβ and GPER. These three receptors with nuclear and extranuclear localization functionally interact with each other and activate many genomic and non-genomic signaling pathways [133]. Importantly, Selective Estrogen Receptor Modulators (SERMs) like tamoxifen and raloxifene, and Selective Estrogen Receptor Downregulators (SERDs) like ICI 182,780 have been identified as GPER agonists [38, 40, 41, 134], resulting in the adjustment or reassessment of studies, in which these SERMs and SERDs were used as agonists/ antagonists and considered to solely target ERα and ERβ. The understanding of GPER`s role in the cardiovascular system comes from studies, which have used GPER selective ligand/ agonist G1. Identification of G15 [46] and generating GPER knockout mice [135] have facilitated in-depth studies to assess the individual roles of those three estrogen receptors ERα, ERβ and GPER within the cardiovascular system [133].

This complexity of estrogen signaling, because of the involvement of three receptors, might explain in part the controversial outcomes of clinical trials, in which conjugated equine estrogens and medroxyprogesterone acetate were used as postmenopausal hormone therapy. These trials reported no beneficial effects of hormone therapy on the cardiovasculature; in fact increased incidence to develop breast cancer and venous thromboembolic events was detected and correlated to hormone therapy [136, 137]. Hence, it is essential to pharmacologically separate estrogen`s beneficial vascular signaling pathways from estrogen`s harmful effects. This might be achieved by using selectively targeting ER specific agonists, which do not elicit the negative side effect of estrogens. GPER agonist raloxifene has recently been shown to minimize the risk of cardiovascular events and breast cancer in younger menopausal women, whereas lasofoxifene had even greater effects [138-141]. However, it is not yet understood if this risk reduction is only due to GPER activation and therefore much more research is needed. Nevertheless, in aging animals, while levels of ERα and ERβ decline, GPER expression is prevalent and mediates estrogen signaling,

suggesting an important role of GPER in postmenopausal women [92]. Further, G1 mimics the beneficial vascular effects of estrogen, without exhibiting the feminizing effects of estrogens and might be an interesting candidate for a new treatment strategy of CVDs in both genders. In summary, all these studies indicate that GPER is responsible for a variety of protective cardiovascular effects and may represent a very interesting target for developing effective treatments for CVDs.

1.7 The Molecular Mechanisms regulating Vascular Cell Function

1.7.1 The TGFβ/ BMP Signaling and its Role on Vascular Cell Function

The superfamily of Transforming Growth Factor β (TGFβ) includes TGFβ1,-2,-3, the Bone Morphogenetic Protein (BMP) subfamily, Growth Differentiation Factors (GDF), activins, inhibins and myostatins. These family members and their downstream pathway components are highly conserved and regulate multiple diverse cellular functions such as growth, adhesion, migration, apoptosis and differentiation [142]. The TGFβs and BMPs have been intensively studied in human vascular diseases and play an important role in regulating vascular homeostasis [143].

TGFβ signaling is mediated via two types of receptors: TGFβ type I and type II transmembrane receptors, both characterized by serine/ threonine kinase activity. In mammals there are five type II receptors and seven type I receptors, which are also termed as activin receptor-like kinases (ALKs). All members of the TGFβ family have to be cleaved by proteases and secreted to become active ligands [144]. Upon binding of the ligand to type II receptors, a heteromeric complex is formed with type I receptors, which is phosphorylated at specific serine and threonine residues in the intracellular juxtamembrane region. This activation leads to the recruitment and phosphorylation of receptor-regulated SMADs (R-SMADs) at two serine residues at their carboxyl termini [145-147]. In most cells, TGFβ signals via TGFβ type II receptor (TβRII) and ALK5. In ECs TGFβ also signals via ALK1, while BMPs elicit their signaling via BMP type II receptor (BMPRII), Activin type II receptors (ActRIIs) and ALK1, 2, 3 and 6 [148, 149]. Furthermore, BMP9 and BMP10 have recently been identified as ligands for ALK1 [150]. ALK4/ 5 phosphorylate R-SMAD2/3, while ALK1/ 2/ 3/ 6 phosphorylate R-SMAD1/5/8. These activated R-SMADs form complexes with SMAD4 and translocate into the nucleus, where, with the help of other transcription factors, they trigger the transcription of their target genes (as depicted in Figure 11) [145-147]. ALK5/ SMAD2/3 signaling induces the expression of fibronectin and

Plasminogen Activator Inhibitor type 1 (PAI-1), which inhibits EC migration and proliferation and thereby prevents capillary-tube formation [149, 151, 152]. ALK1/ SMAD1/5/8 upregulates the expression of Inhibitor of Differentiation 1 (ID-1), leading to increased migration, proliferation and tube formation of ECs [149, 153]. ALK1- and ALK5- pathways elicit opposite responses, but interact with each other to counterbalance and achieve maximal activation [149]. Indeed, as stated by Goumans et al., ALK1 and ALK5 “provide ECs with a TGFβ-dependent switch to fine tune EC function” [154].

The activity of R-SMADs is regulated by inhibitory SMAD6/7 (I-SMAD), which compete with R-SMADs for type I interaction and induce proteosomal degradation or dephosphorylation of R-SMADs, hence inhibiting their signaling (as depicted in Figure 11) [155, 156].

Regulation of the access of TGFβ superfamily ligands to the signaling type I and type II receptors is maintained by soluble ligand binding receptor proteins and by accessory type III receptors. Examples for the soluble ligand-sequestering molecules, preventing TGF superfamily ligand binding, are Chordin/Short gastrulation (SOG), Noggin, Twisted gastrulation (Tsg), Crossveinless-2 (Cv-2), Sclerostin and Follistatin [157]. The most intensively studied accessory type III receptors are endoglin and betaglycan [144, 158], which facilitate the binding of TGFβ and BMPs to their receptors and potentiate their actions [150, 158-160].

Interestingly, independent of SMAD-mediated transcription, the members of the TGFβ superfamily can also activate other cascades that either regulate activation of SMADs or trigger responses, which are unrelated to SMAD-linked signaling. TGFβ can also regulate pathways such as Erk, p38, MAPK, JNK, PI3K/ Akt and small GTPases (rhoA and Cdc42) [161, 162].

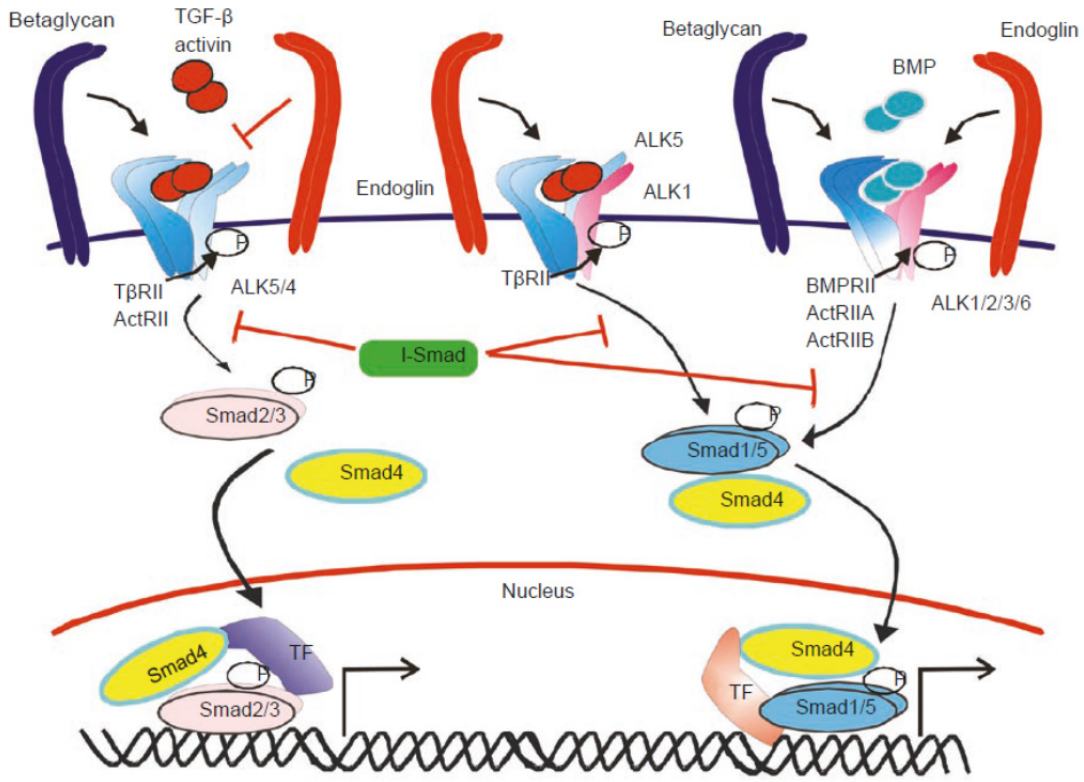

Figure 11: The activation of TGFβ signaling and its downstream effectors [154]. TGFβ: Transforming growth factor β, BMP: Bone morphogenetic protein, TβRII: TGFβ type II receptor, ALK: Activin receptor-like kinases, BMPRII: BMP type II receptor, ActRIIA/ B: Activin type II receptors, I-Smad: Inhibitory Smad, TF: Transcription Factor

The relationship between endothelium and SMCs plays a key role in vascular homeostasis. Recently, it has been shown that the TGFβ superfamily not only mediates effects on the endothelium, but also on VSMCs.

Tight control and balanced coordination between ECs and SMCs are required for the mature vascular network [74], and TGFβ plays a key role in mediating this EC-SMC interaction [163]. While ECs mainly express ALK1, SMCs show higher expression of ALK5 [164], still both receptors have to interact, to fully elicit TGFβ and BMP signaling in the individual vascular cells [151, 165].The essential role of TGFβ within the VSMC-EC interaction was discovered due to mutations of several components in TGFβ-signaling.

Aberrant development of SMCs and defect in primary capillary plexus of the yolk sac was observed in endoglin- and ALK1- deficient mice [144, 148, 166, 167]. Mutations in the genes encoding TβRII, SMAD1/5 or TGFβ1 lead to defects of the vasculature structure or blood vessel organization in mice, indicating deficiency in lining of EC

and SMC development [166]. In summary, TGFβ and BMPs are important regulators of the differentiation and proliferation of SMCs by inducing Matrix-Gla protein (MGP), α -smooth muscle actin (α-SMA) and calponin via ALK1 and ALK5 [165, 168-171].

The role of ALK1/ SMAD1/5/8 pathway within ECs and their function has been intensively studied with contraindicative outcomes. Mostly, it has been shown that upregulated expression of ALK1 and ID-1 correlates with increased proliferation, migration and tube formation of ECs [149, 153, 172, 173], while Mitchell et al. and Lamouille et al. demonstrated that ALK1 acts anti-angiogenic by inhibiting proliferation, migration and re-adhesion of ECs [174, 175]. These opposing results can be explained by the use of different cell types and contexts, but still demonstrate the involvement and essential role of ALK1 in maintaining EC function and angiogenesis.

Furthermore*, in vivo* studies indicated an important role of ALK1/ SMAD1/5/8 signaling in embryonic angiogenesis, since mutations in *SMAD* genes lead to severe defects in embryogenesis and early lethality. SMAD1 and SMAD5 knockout mice died due to allantois defects and lack of placenta formation and SMAD4 mutant mice suffered from lethal cardiovascular defects [176-178].

The importance for intact ALK1/ SMAD1/5/8 signaling is further underlined by the clinical relevance in Hereditary Hemorrhagic Telangiectasia (HHT). HHT is characterized by a loss-of function mutation in ALK1, leading to leaky vessels with impaired endothelial integrity. HHT patients suffer from mucocutaneous telangiectasis, arteriovenous malformations in the brain, lung, liver and gastrointestinal tract, which lead to nose bleeding and gastrointestinal bleeding [154, 179].

In summary, the diversity of ligands, receptors and their various possible combinations reflects the complexity of TGFβ signaling. Furthermore, this signaling and the resulting effects are also context- and cell-type specific, which emphasizes the need of more in-depth studies to elucidate the full spectrum of TGFβ signaling.

1.7.2 The PI3K/ Akt/ NO/ VEGF Pathway and its Role on Vascular Cell Function

The PI3K/ Akt/ NO/ VEGF pathway plays a central role in controlling cell viability by regulating multiple cellular functions such as metabolism, growth, proliferation, survival, transcription, protein synthesis and angiogenesis [180, 181]. This pathway is tightly regulated, but upon dysregulation implicated in several human diseases like diabetes, cancer and CVDs. Therefore, detailed research is necessary for understanding its signaling and may provide new avenues for therapeutic approaches [182].

The phosphatidylinositol-3 kinases (PI3Ks) are highly conserved intracellular lipid kinases, which upon activation phosphorylate phosphatidylinositol and phosphoinositides at their 3`OH group (PIP2→PIP3) [180]. PI3Ks can be grouped into three classes, Class I, II and III, whereby Class I PI3K is prevalent in mammals and stimulated by growth factor Receptor Tyrosine Kinases (RTKs) or GPCRs [183]. Activation of PI3K by growth factors, such as VEGF [184, 185], result in accumulation of signaling proteins with pleckstrin-homology at these activation sites by directly binding to PIP3. These signaling proteins include serine-threonine kinase Akt, also called Protein Kinase B (PKB), and Phosphoinositide-Dependent Kinase 1 (PDK1). The close proximity facilitates PDK1 to phosphorylate and activate Akt [186], leading to phosphorylation of other signaling proteins as well as regulation of glucose homeostasis, cell migration, growth and proliferation [180, 182]. An important signaling protein downstream of PI3K/ Akt is eNOS, which generates NO upon phosphorylation [184, 187]. NO is both, a downstream and an upstream mediator of VEGF-mediated signaling. Under normoxic conditions, NO has been shown to induce VEGF expression [188], hence NO and VEGF stimulate each other in a reciprocal fashion (as depicted in Figure 12). The PI3K signaling is terminated by two different types of phosphatases, Scr-homology 2-containing phosphatase (SHP1 or SHP2) and phosphatase PTEN, which degrade PIP3 to PIP2 and therefore block Akt activation and further signaling [182].

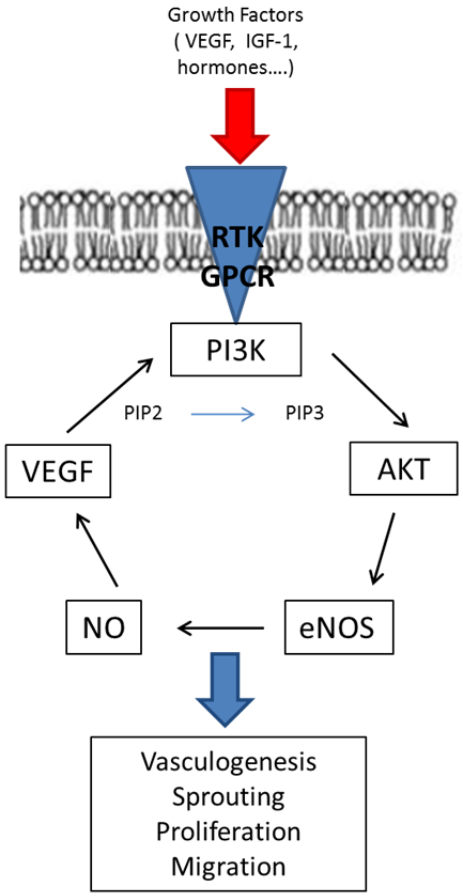

Figure 12: The PI3K/ Akt/ NO/ VEGF pathway. VEGF: Vascular Endothelial Growth Factor, IGF-1: Insulin-like Growth Factor-1, RTK: Receptor Tyrosin Kinase, GPCR: G-protein Coupled Receptor, PI3K: Phosphatidiylinositol-3 Kinase, PIP2: Phophatidylinositol-3,4,5-bisphosphate, PIP3: Phophatidylinositol-3,4,5-trisphosphate, Akt: Protein Serine-Threonine Kinase, eNOS: endothelial Nitric Oxide Synthase, NO: Nitric Oxide

Vascular homeostasis is maintained by a balanced interaction between endothelial and smooth muscle cells. The PI3K/ Akt/ NO/ VEGF pathway has been shown to mediate effects on the endothelium as well as on VSMCs. Tight control and balanced coordination between ECs and SMCs is required for the mature vascular network [74] and several studies reported an important regulatory role of PI3K/ Akt/ NO/ VEGF in postnatal blood vessel formation and processes related to angiogenesis [181, 189-192].

The PI3K/ Akt signaling pathway controls EC viability [180, 181]. Migration and formation of capillary-like structures are essential for EC driven angiogenesis *in vivo*. The protein Akt accumulates in endothelial tip cells with increased migratory abilities [193], resulting in angiogenesis of ECs [181, 189, 190]. Due to its mitogenic and angiogenic role, PI3K/ Akt is also involved in tumorigenesis and has been shown to be highly stimulated in estrogen induced proliferation of ER+ breast cancer cells

[194, 195] and endometrial cancer cells [196]. Indeed, specific inhibitors for PI3K/ Akt are already used clinically as anti-angiogenic agents against cancer [197, 198] and breast cancer [195]. Moreover, PI3K/ Akt has also been shown to control the proliferation of SMCs *in vitro* [199, 200]. Additional confirmation comes from *in vivo* studies, where Akt1 knockout mice showed reduced proliferation, migration and protection against oxidative stress-induced apoptosis of VSMCs [201, 202]. These findings support the essential regulatory role of PI3K/ Akt signaling in vascular cell responses to acute or repetitive injuries, leading to restenosis and atherosclerosis. Additionally, Akt has been shown to regulate the vasomotor tone *in vivo* by inducing NO production. Overexpression of constitutively active Akt increases resting diameter and blood flow in intact animals, while dominant negative Akt inhibits acetylcholine-induced vasodilation [203, 204].

The fundamental regulatory role of VEGF [191] and NO [192] in angiogenesis has also been intensively studied and established. Several studies highlight the importance of VEGF in embryogenesis. Moreover, inactivation of one *VEGF* allele has been shown to result in defective vascularization and embryonic lethality [205, 206]; whereas a Cre-loxP approach to inactivate two isoforms of VEGF in mice resulted in 50% lethality, while the other 50% exhibited impaired myocardial contractility, heart enlargement and onset of ischemic cardiomyopathy [207]. Furthermore, several tumors, including ER+ breast cancer [208-211], show elevated mRNA levels of VEGF, and monoclonal antibodies targeting VEGF are clinically used as anti-cancer agents in therapy [212].

NO angiogenic properties are implicated in promoting survival [213, 214], proliferation [215, 216] and migration [217, 218] of ECs. Studies using eNOS knockout mice revealed impaired morphogenesis and decreased stabilization of angiogenic vessels [219]. Moreover, vasodilation is dependent on endothelium-derived NO, which induces relaxation of SMCs and regulates vascular tone in isolated coronary arteries and vessels. Thereby, NO also stimulates blood flow and decreases systemic blood pressure [220-222]. Additionally, NO inhibits Matrix Metallo Proteinases (MMPs) [223-225], proliferation and migration of SMCs *in vitro*; thus preventing neointima formation after vessel injury [103, 226, 227].

In summary, all these studies provide evidence for the essential role of PI3K/ Akt/ NO/ VEGF pathway in actively controlling cellular functions. This signaling pathway is highly active in cancer and therefore a preferred target of several clinically applied anti-carcinogenic drugs. More importantly, PI3K/ Akt/ NO/ VEGF signaling has been shown to maintain vascular homeostasis and to have beneficial effects on the cardiovascular system. Definitively, more research is needed to fully elucidate the complexity of PI3K/ Akt/ NO/ VEGF signaling and its impact on vasculature. Taken together, this pathway seems to be a promising target and may provide new avenues for therapeutic approaches against CVDs.

2 General Hypothesis and Objectives

The impact of estrogen(s) on the cardiovascular system has been intensively studied; however the mechanism(s) involved remain unclear and subject of intensive research. Protective effects of endogenous estrogens and estrogen replacement therapy against the progression of CVDs in postmenopausal women are supported by multiple epidemiological and animal studies and small clinical trials [78, 79]. This contention was not supported by the results of two large clinical trials i.e. the WHI trial and the HERS [79, 81, 82]. However, re-evaluation of their data demonstrated that, in contrast to older study participants with established cardiovascular pathology, estrogen replacement therapy was effective and protective in younger healthy women [81, 82]. More importantly, the timing of estrogen therapy initiation following menopause within five years of reaching menopause seems to play a decisive role in defining its protective actions on the vasculature. The beneficial actions of estrogen on the endothelium [102] and it`s anti-mitogenic actions on VSMCs proliferation [105, 106, 228, 229] play an important role in cardiovascular protection. These differential effects of estrogens on ECs and SMCs are potentially mediated by three different estrogen receptors, ERα, ERβ and GPER, which are expressed in vascular cells [112, 113, 230, 231]. Studies with ERα/ ERβ double knockout mice reported decreased proliferation of VSMCs upon estrogen treatment [123]; moreover we observed that capillary stimulating effects of E2 were mimicked by BSA-tagged E2, which is not membrane permeable [100]. Based on these observations, we hypothesize that the vascular actions of E2 may be potentially mediated via the newly discovered membrane bound GPER.

The **first aim of this study** was to investigate the **impact of GPER on endothelial cell function**. To accomplish this, we assessed vasculogenesis, sprouting, migration and proliferation of HUVECs in response to the GPER specific agonist G1, the GPER specific antagonist G15 and to GPER silencing with specific siRNA.

We further investigated and identified molecular mechanisms for GPER-induced vasculogenesis. Findings that mutations in *ALK1* and *SMADs* lead to severe defects in embryogenesis and early lethality [176-178], suggest that ALK1/ SMAD1/5/8 signaling plays a key role in embryonic angiogenesis. Thus, the **second aim** was to investigate and dissect the **role of GPER activation on ALK1/ SMAD1/5/8**

signaling and whether **ALK1/ SMAD1/5/8 signaling mediates GPER-induced capillary formation by HUVECs.**

A classical GPCR mechanism of action is the activation of the enzyme adenylyl cyclase and the production of cAMP [232], moreover treatment with estrogen has been shown to induce cAMP in cells [104, 233-236]. Additionally, membrane-permeable cAMP analogues have been reported to induce angiogenesis [191, 237-239]. Hence, the **third aim** was to assess whether **GPER activation induces cAMP generation in HUVECs** and whether **cAMP signaling modulates GPER-stimulated capillary formation and ALK1/ SMAD1/5/8 signaling in these cells.**

Several studies have reported an important regulatory role of PI3K/ Akt in postnatal blood vessel formation and processes related to angiogenesis [181, 189, 190]. Furthermore, estrogen-stimulated angiogenesis has been shown to be mediated via the PI3K/ Akt pathway [100, 240, 241]. Hence, the **fourth aim** was to assess whether **GPER activation induces PI3K/ Akt signaling and whether this pathway regulates GPER-mediated capillary formation**. **Moreover, we investigated whether there is a crosstalk between these two pro-angiogenic pathways PI3K/ Akt and ALK1/ SMAD1/5/8 in HUVECs, following GPER activation.**

Since abnormal growth of smooth muscle cells contributes to neointima formation, our **fifth aim** was to investigate whether **GPER activation alters proliferation and migration of HCASMCs;** and whether the growth modulatory effects of GPER in HCASMCs are mediated via ALK1/ SMAD1/5/8 and/ or PI3K/ Akt signaling.

Overall, the findings of the present study will help in understanding the mechanism by which GPER influences vascular cells and subsequently the cardiovascular system. In addition, these findings would help to develop improved hormone replacement therapy for menopause associated cardiovascular disease.

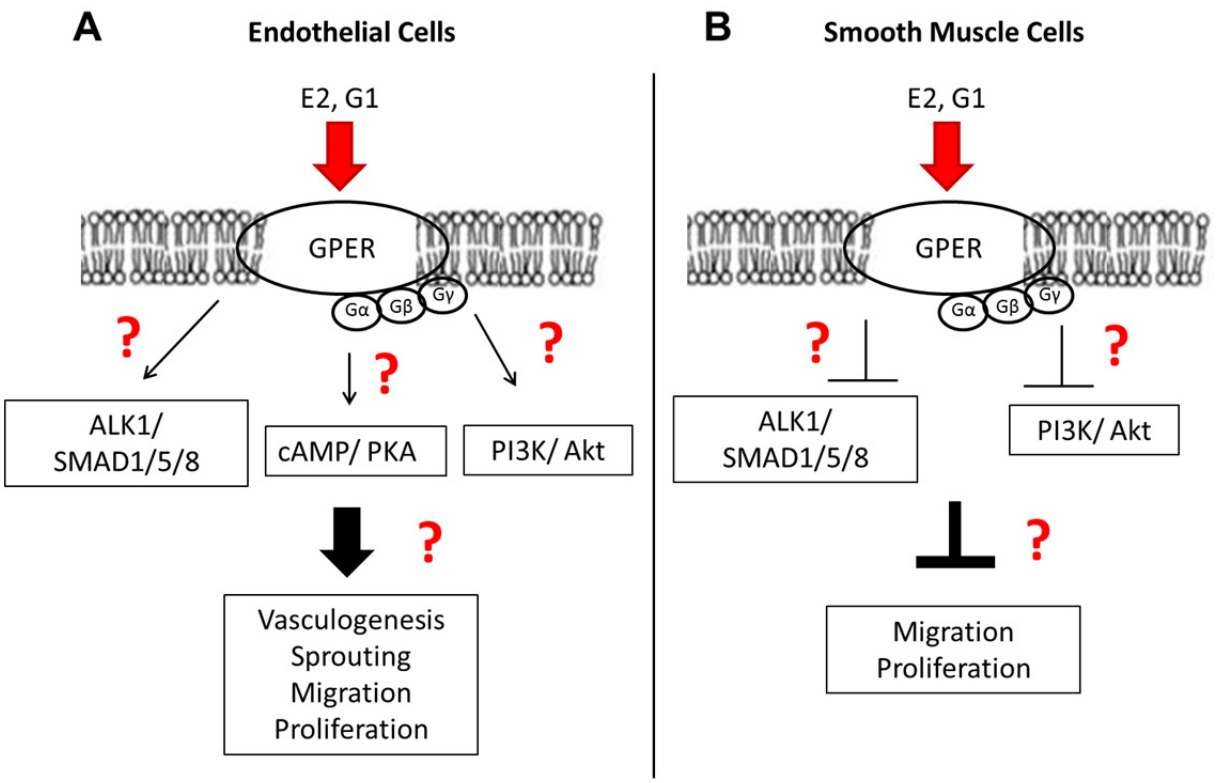

Figure 13: Schematic Depicting the Aims of the Study. **Panel A:** The impact of GPER on endothelial cell function and the possible molecular mechanisms leading to GPER-induced improvement of endothelial function. We used HUVECs as our cell model. **Panel B:** The impact of GPER on smooth muscle cell function and the possible molecular mechanisms mediating GPER`s effect on proliferation and migration of SMCs. We used HCASMCs as our cell model.

3 Materials and Methods

3.1 Materials

3.1.1 Cell Culture

Antibioticum-Antimycotium	Life Technologies, Carlsbad, CA, USA (15240-096)
Cell culture flasks, dishes, plates	TPP, Trasadingen, CH
Collagen (rat tail)	Roche Diagnostics, Mannheim, DE (11179179001)
Cryotubes	Nalge Nunc International, USA (377224)
DMEM/F12	Sigma-Aldrich, St. Louis, USA (21331-020)
EBM-2 Basal Medium	Lonza, Walkersville, USA (CC-3156)
ECFC	Lonza, Walkersville, USA (00189423)
EGM-2 SingleQuots	Lonza, Walkersville, USA (CC-4176)
FCS Charcoal Stripped	Thermoscientific, Waltham, MA, USA (SH30068.03)
FCS	Thermoscientific, Waltham, MA, USA (SH30070)
HBSS (without Ca^{2+} and Mg^{2+})	Bioconcept, Allschwill, CH (3-02K34-I)
HCASMCs	Life Technologies, CA, USA (C-017-5C)
HUVEC	Lonza, Walkersville, USA (CC-2517)
L-Glutamine	Life Technologies, Carlsbad, CA, USA (25030-024)
LSGS	Life Technologies, Carlsbad, Ca, USA (S003-10)
PBS tablets	Gibco BRL, Paisley, UK (18912-014)
Reaction tubes (1.5ml), tips	Eppendorf, Hamburg, DE
SMGS	Cascade Biologics, Inc, USA (S-007-25)
Trypsin	Sigma-Aldrich, St. Louis, USA (T-3924)

3.1.2 Cell Counting

Coulter Isoton II diluent	Kantonsapotheke, Zürich, CH
Coulter Clenz cleaning agent	Kantonsapotheke, Zürich, CH

3.1.3 Antibodies and Peptides

Primary Antibodies

Anti- β-Actin	Sigma, St. Louis, USA (A5441)
Anti-Akt	Cell Signaling Technology, MA, USA (9272)

Anti-ALK1	Santa Cruz, Dallas, Texas, USA (sc-28976)
Anti-ALK5	GeneTex, Taiwan, R.O.C. (GTX102784)
Anti-BMP2/4	Santa Cruz, Dallas, Texas, USA (sc-137087)
Anti-enos	Cell Signaling Technology, MA, USA (9586)
Anti-GPER	Santa Cruz, Dallas, Texas, USA (sc-48525-R)
Anti-Id1	Abcam, Cambrige, UK (ab66495)
Anti-MEK	Cell Signaling Technology, MA, USA (9122)
Anti-PAI-1	Millipore, Temecula, USA (09-726)
Anti-pAKT (Ser473)	Cell Signaling Technology, MA, USA (9271)
Anti-penos (Ser113)	Cell Signaling Technology, MA, USA (9575)
Anti-pMEK (Ser217/221)	Cell Signaling Technology, MA, USA (9121)
Anti-pSMAD1/5/8 (Ser463/465)	Millipore, Temecula, CA (AB3848)
Anti-Smad1/5/8	Abcam, Cambrige, UK (ab13723)
Anti-VEGF A	Santa Cruz, Dallas, Texas, USA (sc-152)

Secondary Antibodies

Goat anti-Mouse IGF-Peroxidase Conj.	Pierce, Rockford, USA (31430)
Goat anti-Rabbit IGF-Peroxidase Conj.	Pierce, Rockford, USA (31460)
IRDye 680 Conj. Goat anti-Mouse IgG	LI-COR, Nebraska, USA (926-32220)
IRDye 800 Conj. Goat anti-Rabbit IgG	LI-COR, Nebraska, USA (926-32211)

Peptides

Human VEGF antibody	R&D Systems, Minneapolis, USA (MAB293)
PDGF	Sigma-Aldrich, St. Louis, USA (P-3201)
	R&D Systems, Minneapolis, USA (370-AL-100)
Recombinant Human ALK1 Fc Chimera	
Recombinant Human BMP2	Sigma-Aldrich, St. Louis, USA (H4791)
Recombinant Human BMP9	R&D Systems, Minneapolis, USA (3209-BP)
Recombinant Human EGF	Sigma-Aldrich, St. Louis, USA (E9644)
Recombinant Human TGFβ	R&D Systems, Minneapolis, USA (240-B)

3.1.4 **RT-PCR**

Quick-RNA Mini Prep	Zymo Research, USA (R1055)
GoTaq 2-Step RT-qPCR System	Promega, Wisconsin, USA (A6010)
DEPC-treated water	Life technologies, CA, USA

3.1.5 **Primers**

18sRNA_For	AAGAGAGCCGAGGCAATTACC
18sRNA_Rev	GCTCGCATTTTGAGGCTGTTG
VEGFa_For	CATGCAGATTATGCGGATCAAAC
VEGFa_Rev	GGTCTGCATTCACATTTGTTGTG

Self designed Primer and purchased at Microsynth, Balgach, Switzerland

3.1.6 **Chemicals and Buffers**

10x Tris/Glycine Buffer	Bio-Rad, Reinach, CH (161-0771)
10x Tris/Glycine/SDS Buffer	Bio-Rad, Reinach, CH (161-0772)
5x siRNA Buffer	Dharmacon, Lafayette, USA (B-002000-UB-100)
Actinomycin D	Calbiochem, San Diego, USA (114666)
Ammonium Persulfate (APS)	Sigma-Aldrich, St. Louis, USA (A6761)
Aprotinin	Sigma-Aldrich, St. Louis, USA (A-1153)
Batimastat	Sigma-Aldrich, St. Louis, USA (SML0041)
BCA Protein Assay Kit	Pierce, USA (23227)
BD Matrigel	BD Biosciences, Franklin Lakes, USA (356237)
Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, USA (A-3059)
Cell Lysis Buffer	Cell Signaling, Danvers, USA (9803)
Cycloheximide	Sigma-Aldrich, St.Louis, USA (PS1002)
DAF-2-DA	Sigma-Aldrich, St.Louis, USA (D225)
DDA	Sigma-Aldrich, St.Louis, USA (D1285)
DBcAMP	Sigma-Aldrich, St.Louis, USA (D0627)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, St.Louis, USA (41640)
Ethanol 70%	Kantonsapotheke Zurich
Ethanol 100%	Kantonsapotheke Zurich
17-β-estradiol (E2)	Steraloids, Newport, USA (E950)

Fibrinogen Type I	Sigma-Aldirch, St.Louis, USA (F0895)
Fluo-4, AM	Molecular Probes, Eugene, OR, USA (F14201)
Flutamide	Sigma-Aldrich, St.Louis, USA (F9397)
G1	Calbiochem, Darmstadt, Germany (371705)
G15	Sigma-Aldrich, St. Louis, USA (G6548)
Hyperfilm ECL	Amersham, Dübendorf, CH (RPN2103K)
ICI 182-780	Tocris, Bristol, UK(1047)
Loading Butter (5x)	Fermentas, Burlington, Canada (R0891)
LY0294002	Calbiochem, Merck, Darmstadt, D (440202)
L-NAME	Sigma-Aldrich, St.Louis, USA (N5751)
L-NMMA	Sigma-Aldrich, St.Louis, USA (M7033)
Methanol	Sigma-Aldrich, St.Louis, USA (32213)
MPP	Tocris, Bristol, UK (1991)
mm-IBMX	Calbiochem, San Diego, USA (454202)
Noggin	R&D Systems, Minneapolis, MN, USA (6057-NG-025)
PDGF	Sigma-Aldrich, St.Louis, USA (P-3201)
Pertussis Toxin	Sigma-Aldrich, St.Louis, USA (P7208)
PKI (5-24)	Santa Cruz, Dallas, Texas, USA (CAS 99534-03-9)
Ponceau S Solution (2%)	Sigma-Aldrich, St.Louis, USA (P-7767)
Precision Plus Dual Color Standard	Biorad, Reinach CH (161-0374)
SJN2511	Tocris, Ballwin, MO, USA (3211)
Sodium Chloride (NaCl)	Sigma-Aldrich, St.Louis, USA (71381)
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, St.Louis, USA (L-5750)
Sodium Hydroxyde (NaOH)	Sigma-Aldrich, St.Louis, USA (S-8045)
SuperSignal West Dura	Pierce, Rockford, USA (34075)
SuperSignal West Pico	Pierce, Rockford, USA (34080)
Temed	Sigma-Aldrich, St.Louis, USA (T-9281)
Thrombin	Sigma-Aldrich, St.Louis, USA (T-4648)
Triton X-100	Sigma-Aldrich, St.Louis, USA (X100)
Tween 20	Sigma-Aldrich, St.Louis, USA (P-7949)

Cytodex beads 3	GE Healthcare, Uppsala, Sweden (17-0485-01)
DharmaFECT 4	Fischer Scientific, Wohlen, CH (T-2004-03)
0.5M Tris/HCl pH 6.8	Biorad, Reinach, CH (161-0799)
1.5M Tris/HCl pH 8.8	Biorad, Reinach, CH (161-0799)
Rotiphorese Gel 30	Carl Roth GmbH, Karlsruhe, D (3029.1)

3.1.7 **siRNAs**

On-TARGETplus non-targeting Pool, Thermo Scientific Dharmacon, Lafayette, USA (D-001810-10-20)

(UGGUUUACAUGUCGACUAA,

UGGUUUACAUGUUGUGUGA,

UGGUUUACAUGUUUUCUGA,

UGGUUUACAUGUUUUCCUA)

On-TargetplusSMARTPool GPER1, Thermo Scientific Dharmacon, Lafayette, USA (L-005563-00-0005)

(GGGUGAAGCGCCUCAGUUA,

AACAGAAGCAGGCCUCGUC,

CCAGUCGUGAGGUUUCCUA,

UAGCGGUCGAAGCUCAUCC)

On-TARGETplusSMARTpool Smad1, Thermo Scientific Dharmacon, Lafayette, USA (L-012723-00-0005)

(GCUCUAUUGUCUACUAUGA,

GGCGGUUGCUUAUGAGGAA,

CAACAAUCGUGUGGGUGAA,

CAAAUGGGUUCACCUCAUA)

3.1.8 **ELISA**

Direct cAMP ELISA kit	Enzo Life Sciences, Lausen, Switzerland (ADI-900-066)

3.1.9 **Instruments and Software**

5417R Centrifuge	Eppendorf, Hamburg, DE
90SE Ultracentrifuge, Sorvall	Thermo Fisher Scientific Inc, USA

Bio-Rad CFX Real-Time PCR Detection System	BioRad
Coulter Z1, Cell Counter	Coulter Electronics, Luton, UK
H54AR Precision balance	Mettler-Toledo, USA
Hera Cell 150, Cell incubator	Thermo electron corporation,
Image Studio Lite	LI-COR, Nebraska, USA
ImageJ	
LI-COR Odyssey 3.0 Infrared Imaging System	LI-COR, Nebraska, USA
Magellan 6	Tecan, Salzburg, Austria
Mini-PROTEAN 3 Electrophoresis Cell	Biorad, Reinach, CH
Mini-Trans-Blot Electrophoretic Transfer Cell	Biorad, Reinach, CH
NanoDrop system	Tecan, Salzburg, Austria
Odyssey 3.0	LI-COR, Nebraska, USA
Olympus BX61 Microscope	Olympus, Volketswil, CH
Olympus CKX41 Microscope, Bright field	Olympus, Volketswil, CH
Olympus IX81 Microscope, Fluorescence	Olympus, Volketswil, CH
Power Pac 200, Power Supplier	Biorad, Reinach, CH
Rotina 46R Centrifuge	Hettich, Bäch, CH
Sonicator	Bandelin electronics, Berlin, D
StatView, Version 5.0.1	SAS Institute, Cary, USA
Tecan Infinite series M200	Tecan, Salzburg, Austria
Xcellence pro	Olympus, Volketswil, CH

3.2 **Methods**

3.2.1 **Cell Culture**

Human Umbilical Vein Endothelial Cells (HUVECs) were cultivated up to passage 9 in 75 cm^2 flasks with HUVECs complete growing medium (DMEMF12, supplemented with L-Glutamine, LSGS, Antibioticum- Antimycoticum (AA) and 10% FCS) under standard tissue culture conditions (5% CO_2 at 37°). Medium was renewed every two days and upon confluency cells were washed with HBSS $-Ca^{2+}$/ $-Mg^{2+}$, trypsinized for two minutes at 37°C and neutralized in the double amount of the complete medium, finally centrifuged at 1200rpm for five minutes and re-plated at a split of 1:4 in flasks,

culture dishes or well-plates. For experiments, HUVECs were starved ON with HUVECs starving media (DMEMF12, supplemented with L-Glutamine, AA and 1% BSA) and also treated within this starving media. For vasculogenesis studies DMEMF12, supplemented with L-Glutamine, AA and 0.4% BSA was used.

Endothelial Colony Forming Cells (ECFCs) were cultivated up to passage 8 in Collagen-coated 75 cm^2 flasks with ECFC complete growing medium (EGM-2, supplemented with SingleQuots, AA and 10 % FCS) under standard tissue culture conditions (5% CO_2 at 37°C). Medium was renewed every two days and upon confluency cells were washed with HBSS $-Ca^{2+}$/ $-Mg^{2+}$, trypsinized for two minutes at 37°C and neutralized in the double amount of the complete medium, finally centrifuged at 1200rpm for five minutes and re-plated at a split of 1:4 in flasks, culture dishes or well-plates. For experiments, ECFCs were starved ON with ECFCs starving media (EGM-2, supplemented with AA and 1% BSA) and also treated within this starving media. For vasculogenesis studies EGM-2, supplemented with AA and 0.4% BSA was used.

Human Coronary Artery Smooth Muscle Cells (HCASMCs) were cultivated up to passage 8 in 75 cm^2 flasks in HCASMCs complete growing medium (M231, supplemented with SMGS and AA) under standard tissue culture conditions (5% CO_2 at 37°C). Medium was renewed every two days and upon confluency cells were washed with HBSS $-Ca^{2+}$/$-Mg^{2+}$, trypsinized for two minutes at 37°C and neutralized in the double amount of the complete medium and re-plated at a split of 1:4 in flasks, culture dishes or well-plates. For experiments, HCASMCs were starved ON in HCASMCs starving media (M231, supplemented with AA and 1% BSA) and also treated within this starving media.

Cryopreservation of Cells

Confluent cells were trypsinized and centrifuged as described above. The resulting cell pellet was resuspended in 4 ml ice-cold complete growing medium containing 10% DMSO, serving as cryoprotective agent. Aliquots of 1 ml, in cryotubes, were gradually frozen to –80°C in a Mr. Frosty box (freezing container filled with 2-propanol). For long-term storage the cells were kept in liquid nitrogen (-196°C).

Cell Defrosting

HUVECs, ECFCs and HCASMCs in cryotubes were taken from the liquid nitrogen tank and rapidly thawed within a warm water bath. The cell suspension was slowly transferred into a 75 cm2 culture flasks, containing 10 ml of the respective complete growing medium and incubated under standard tissue culture conditions. The medium was renewed after 24 hours.

3.2.2 **Vasculogenesis Studies**

To assess EC- induced microvessel formation, vasculogenesis studies were performed with HUVECs and ECFCs. HUVECs and ECFCs were trypsinized and treated with various experimental agents, 30 minutes pre-treatment with antagonist or inhibitors, followed by 30 minutes with agonists or ligands. Medium, used for HUVECs and ECFCs, was DMF12, supplemented with L-Glutamine, AA and 0.4% BSA or EBM2, supplemented with AA and 0.4% BSA, respectively.

Aliquots of 50 µl, containing 4000 cells, were transferred onto µ-slides, coated with Matrigel and incubated ON in 5% CO_2 at 37°C. After ON incubation, microvessel formation was analyzed using an Olympus inverted microscope (CKX41, 10x magnification), with which photomicrographs were taken. Capillary length was randomly measured at three separate locations using the Xcellence pro-software (Olympus) and the average compared to the untreated control. DMSO at a final concentration of 0.1% was used as untreated (vehicle) control.

3.2.3 **Sprout Formation Studies**

To investigate whether our treatments induce sprout formation in HUVECs Cytodex3 beads in fibrin gels were used. HUVEC-coated beads were resuspended in a fibrinogen/ aprotinin-solution (500 beads/ ml). Per well of a 24-well plate 0,625 U/ ml of Thrombin/ was added, followed by 0.5 ml of beads/ fibrinogen/ aprotinin-solution and gently mixing. For complete polymerization, the plate was incubated for five minutes at RT followed by 15 minutes at 37°C. For the treatment antagonists were applied 30 minutes prior to agonists in 500 µl of DMF12, supplemented with L-Glutamine, AA and 0.4% BSA. After four days photomicrographs of the gels were taken using the Olympus inverted microscope (10x magnification) and sprouting number was measured using the Xcellence pro-software.

3.2.4 **Migration Studies**

To investigate whether migration of HUVECs was induced with our treatments or migration of HCASMCs was inhibited, a scratch assay was performed. Therefore cells were grown until confluency in 24-well plates. After ON starvation with starving media, a scratch was performed using the blue pipette tip, followed by 2x washing with PBS to remove swimming cells. For the treatment antagonists were applied 30 minutes prior to agonists, within this starving media. In HCASMCs the starving media for the experiment additionally contained 20 ng/ml of Platelet Derived Growth Factor (PDGF) to induce migration.

Photomicrographs were taken using the Olympus inverted microscope at timepoint 0, when scratch was performed, and at timepoint 1, after 24 hours of incubation and wound area was measured using the Xcellence pro-software and values calculated accordingly: (area T1- area T0)/ area T0.

3.2.5 **Cell Proliferation Studies**

Effect of our treatments on cell proliferation of HUVECs or HCASMCs was assessed by counting the cell number. Plating was performed as follows: 20 000cells/ well for HUVECs and 80 000cells/ well for HCASMCs in a 12-well plate on day-2. On day -1 the cells were starved using starving medium ON and on day 0 treatment was performed within the same media, antagonists were applied 30min prior to agonists. In HCASMCs the starving media for the experiment additionally contained 20 ng/ml of PDGF to induce proliferation. Treatment was renewed every two days and lasted four days in HUVECs and six days in HCASMCs. To stop the experiment cells were washed with HBSS $-Ca^{2+}$ / $-Mg^{2}$ and trypsinized with 200 µl for five minutes. This suspension was transferred into cuvettes and cell number assessed using the Coulter Counter.

3.2.6 **Protein Expression Studies**

Expression of GPER, ALK1, ID-1, ALK5, PAI-1, β-Actin, phosphorylation of SMAD1/5/8, AKT, eNOS and release of BMP2 and VEGF A were analyzed by Western Blotting. Briefly HUVECs, ECFCs and HCASMCs were grown till confluency in 28 cm^2-dishes and were starved ON. Subsequently cells were treated for 30 min with antagonists or inhibitors, followed by 45 minutes agonists within starvation media, if not stated otherwise. For the actual experiment time in HCASMCs, the starvation media contained 20 ng/ml PDGF. Cells were washed with cold PBS and

lysed in 50 µl Lysis Buffer. Protein concentration was assessed using the BCA protein assay kit, 30 µg of protein was mixed with Laemmli Sample Buffer and DTT, incubated for five minutes at 95°C and resolved electrophoretically with 10% SDS-gels. Separated proteins were transferred onto nitrocellulose membranes at 32 A during 60 minutes. To prevent unspecific binding, membranes were blocked using 5% milk in PBS-Tween. Primary Antibody for protein of interest was added onto membrane in 1:1000 dilution in 5% BSA in PBS-Tween and incubated ON at 4°C, subsequently followed by incubation of secondary LICOR- or HRP-anchored antibody, with 1:10 000 and 1:25 000 dilution, respectively, in 1% milk in PBS-Tween, for 1 hour at RT. After washing for 30 minutes with PBS-T, the blots were analyzed with LI-COR system or were covered with SuperSignal West Dura LuminolSubstrate for five minutes and then exposed to Hyperfilm ECL films.

Membrane Fractionation

HUVECs were grown till confluency in 28 cm^2-dishes and harvested using 50 µl Lysis Buffer. This cell suspension was centrifuged at 1000 g for 10 min at 4°C, resulting supernatant was transferred into new Eppi, diluted with 4 ml of Lysis Buffer and centrifuged 10'000 g for 1 hour at 4°C using the ultracentrifuge. This time the resulting supernatant was discarded, as it contained cytosolic fraction, and the remaining pellet, containing membrane fraction, resuspended in 50 µl Lysis Buffer and used for BCA and Western Blotting, as described in *Protein Expression Studies*.

Protein Release Studies

Release of BMP2 into the supernatant of HUVECs was assessed using Western Blotting. HUVECs were grown till confluency in 28 cm^2-dishes and were starved ON. HUVECS were washed twice with PBS and treated with the antagonist for 30 min, followed by another 30 min with the agonist within the volume of 200 µl plain DMEMF12. Supernatant was collected and cells were lysed as described above. 24 µl of supernatants and 30 µg of cell-lysate protein were separated electrophoretically in 12% SDS-gels. Further steps were performed according to protocol, already described in *Protein Expression Studies*.

3.2.7 **NO Measurement**

Generation of NO by HUVECs was measured, using DAF-2DA, as it`s cleavage and increased fluorescence is correlating with increased NO levels within the cells.

HUVECs were seeded in 6-well plates, grown until confluency and starved ON using starving medium. DAF-2DA was added to a final concentration of 10 µmol/L and incubated for 1 hour at Room Temperature (RT) in the absence of light. Subsequently cells were washed 2x with HBSS and treated with G1 or E2 for 15 min within the starving media. NO generation was measured at 450 nm at the TECAN plate reader.

3.2.8 **siRNA Transfection**

HUVECs were grown in 28 cm^2-dishes till 60- 70% confluency, followed by ON starvation with DMEMF12, supplemented with L-Glutamine, LSGS, 1% BSA but without AA prior to the transfection. GPER, Smad1 and non-targeting On-Target Plus Smart Pool siRNA were applied with a final concentration of 50 nmol/L, using 5 µmol/ L Dharmafect. siRNA stock solutions (100 µmol/L) were diluted in 1x Buffer. DMEMF12, supplemented with L-Glutamine, was used to prepare siRNA and Dharmafect solutions and incubated for five minutes at RT, followed by combining both solutions and incubation for 20 minutes at RT. The solutions were transferred onto the cells and growing media, without AA, was added on top. After 24 hours of incubation with the transfecting agents, the medium was replaced to complete growing medium. Silencing of the target genes was observed after 72 hours of transfection, which was confirmed by Western blotting. Further experiments were conducted after those 72 hours. For *Protein Expression Studies* transfected cells were pre-starved for four hours, followed by according treatment and lysis. For *vasculogenesis studies* transfected cells were trypsinized after 72 hours, and the procedure applied, as described in *vasculogenesis studies*.

HCASMCs were plated in a density of 80 000 cells/ well in a 12-well plate, followed by starvation ON the next day with DMEMF12, supplemented with L-Glutamine, LSGS, 1% BSA and without AA prior to transfection. GPER and non-targeting On-Target Plus Smart Pool siRNA were applied with a final concentration of 50 nmol/L using 5 µmol/L Dharmafect. siRNA stock solutions (100 µmol/L) were diluted in 1x Buffer. DMEMF12, supplemented with L-Glutamine, was used to prepare siRNA and Dharmafect solutions and incubated for five minutes at RT, followed by combining both solutions and incubation for 20 minutes at RT. The solutions were transferred onto the cells in the morning and growing media, without AA, was added on top. After 24 hours of incubation with the transfecting agents, the medium was replaced to

complete growing medium. Silencing of the target genes was observed after 72 hours of transfection, which was confirmed by Western blotting.

To assess cell proliferation within GPER silenced HCASMCs media was changed in the evening of transfection day to HCASMCs starvation medium, containing 20 ng/ml PDGF and the agonist. Treatment media was renewed every day and cell counting performed on day 3, 72 hours after transfection as described in the *Cell proliferation studies.*

3.2.9 **RT-PCR Gene Expression Studies**

To asses gene expression quantitative PCR was performed using total RNA, extracted from HUVECs. These cells were grown in 28 cm^2-dishes, starved ON using starving media and treated with antagonist for 30 minutes, followed by the agonist for 45 minutes within the same media. To perform RNA extraction the Quick-RNA MiniPrep Kit was used according to the protocol. Briefly the cells were dissolved in 300 µl RNA- Lysis Buffer, followed by many centrifugation steps, one DNA digesting step and many washing steps. Finally the purified RNA was eluted with 30 µl DNase/ RNase free water at top speed for 30 seconds, and then quantified with the NanoDrop-Tool of the Tecan Platereader. Synthesis of cDNA and determining mRNA expression levels were performed with the GoTaq 2-Step RT-qPCR System using 50 ng/µl of RNA and according to the protocol. In table 1 you see the program of thermo cycles used for synthesizing cDNA.

Step	Temperature	Time
Annealing	25°	10 min
Extending	48°	30 min
Inactivating	98°	5 min
Chill	4°	/

Table 1: Program of the Thermo cycler for cDNA synthesis

Transcribed cDNA was then diluted accordingly to the necessary amount (1:4 in DEPC treated water) and mixed with GoTaq Mastermix, the Forward and the Reverse Primer, according to the Protocol. Table 2 shows the Program of the Thermo Cycler for the quantitative PCR.

Repetition	Temperature	Time
1	95°	2 min
1	95°	3 sec
40	60°	30 sec

Table 2: Program of the Thermo cyclcer for quantitative cDNA synthesis

The GoTaq 2-Step RT-qPCR System was used for determining gene expression values of VEGF-A by cycle threshold levels and normalized to 18s RNA. The sequence of these self-designed primers was chosen out of literature and is listed in Table 3.

Primer	sequence
FWD VEGF A	CATGCAGATTATGCGGATCAAAC
REV VEGF A	GGTCTGCATTCACATTTGTTGTG
FWD 18 sRNA	AAGAGAGCCGAGGCAATTACC
REV 18 sRNA	GCTCGCATTTTGAGGCTGTTG

Table 3: Sequences of Primers, used for RT-PCR

3.2.10 **ELISA cAMP Measurment**

Generation of cAMP in HUVECs was assessed using a direct cAMP ELISA kit. Cells were plated in a density of 400 000 cells/ well in a 6-well plate and starved ON the following day. Treatment was performed accordingly: 10 minutes with PDE-inhibitor mm-IBMX, followed by antagonist or vehicle for another 10 minutes and finally 15 minutes with agonist or vehicle, all in DMEMF12, supplemented with L-Glutamine. Supernatant was stored for eventual further analyzing at -80°C and the cells lysed with 300 µl of 0.1 M HCl+0.1% Triton X-100 for 20 minutes. As the samples were acetylated, we also applied the acetylation step at the standards of the kit. All further steps were performed according to the protocol. Finally the optical density was measured at 405 nm with the Tecan platereader and analyzed using the Magellan software. The obtained values of cAMP (pmol/ ml) were normalized to the corresponding total protein concentration, assessed with the BCA protein assay kit.

3.2.11 **Statistical Analysis**

Data was analyzed using ANOVA and statistical significance ($p<0.05$) was calculated according to Fisher's Least Significant Difference test.

4 Results

4.1 GPER Activation improves Endothelial Cell Function

Objective

The beneficial effects of estrogens on the endothelium have been well established, however, the role of GPER in mediating these effects remains unclear. Hence, under this specific aim, we examined the impact of GPER activation on EC function.

Introduction

Initiation of atherosclerosis and subsequent progression of CVDs is associated with both anatomical and functional disruption of vascular endothelium. [63]. Moreover, endothelial repair or regeneration by local ECs or by circulating ECFCs is suggested to protect against vascular remodeling associated with atherosclerosis and CVDs [242-244].

Results from several *in vivo* studies provide strong evidence in support for the vasoprotective actions of estrogens. Indeed, estradiol significantly inhibits injury-induced and pathological vascular remodeling processes [92]; induces endothelial growth/ proliferation, migration[102, 245] and capillary formation [99] and improves endothelium-dependent vascular relaxation. The formation of capillaries (angiogenesis or neovascularization) is a carefully balanced process controlled by multiple growth factors and signalling pathways. RTK activators like VEGF, Hepatocyte Growth Factor (HGF), Stromal Derived growth Factor-1 (SDF-1), second messengers like NO, calcium and cAMP are important molecules that stimulate ECs to form capillaries [191, 192, 246, 247].

Based on our previous finding that the capillary stimulating effects of E2 are mimicked by its non-permeable analogue, BSA-tagged E2 [100], we hypothesize that these actions are potentially mediated via the newly discovered membrane bound GPER. In this study, we assessed the effects of GPER activation on EC function. We addressed this question using several *in vitro* techniques to study vasculogenesis, migration and proliferation of HUVECs.

Methods

As described in section 3.2.

Results

4.1.1 GPER Activation induces Capillary Formation

To investigate the effects on vasculogenesis we used two different assays. The presence of GPER in HUVECs was confirmed by Western Blotting of membrane fractions, isolated by ultracentrifugation from HUVECs. Briefly, HUVECs, expressing GPER (Figure 14A) in the cell membrane, were employed to study capillary formation and sprouting, using a 2D-matrigel based assay and a Cytodex bead assay, respectively.

In cells treated with either the GPER specific agonist G1 (10 nmol/L) or 17-β estradiol (E2; 10 nmol/L), capillary formation increased from 100±22 % to 210±41% and 517±46%, respectively ($p<0.05$ relative to control; Figure 14B). The stimulatory effects of G1 on capillary formation were abrogated in the presence of the GPER-specific antagonist G15 (100 nmol/L) and the ER unspecific antagonist ICI 182,780 (100 nmol/L). G1-induced capillary formation was reduced from 210±41% to 52±5.8% and 63±9%, respectively, whereas E2-induced capillary formation was reduced from 517±46% to 67±12.4% and 72±7.5%, respectively ($p<0.05$ relative G1 or E2 treated HUVECs; Figure 1B). Taken together our findings provide evidence for a specific role of GPER in inducing capillary formation by HUVECs.

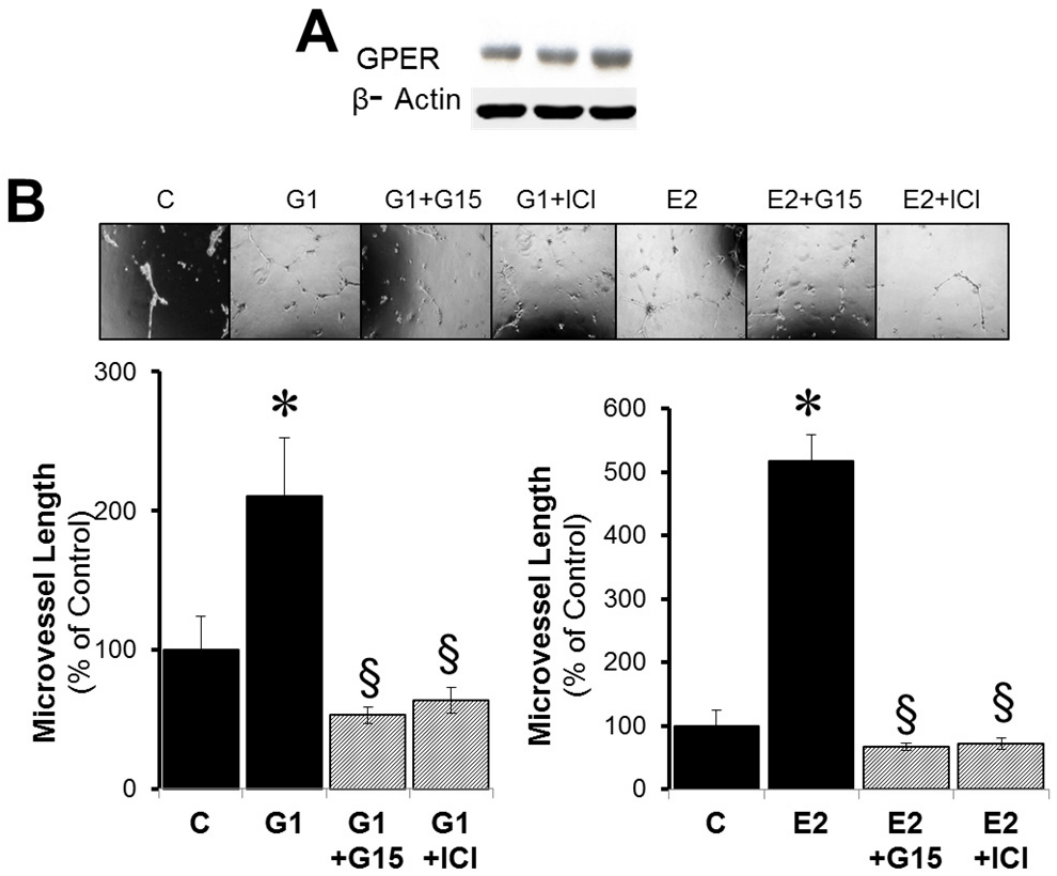

Figure 14. Panel A, Representative Western Blot, showing that HUVECs express GPER in the cell membrane preparations. **Panel B,** Bar graphs and representative photomicrographs showing the effects on capillary formation by HUVECs. The cells were treated with GPER specific agonist (G1; 10 nmol/L) or estradiol (E2; 10 nmol/L) in the presence or absence of GPER specific antagonist (G15; 100 nmol/L) or ER-unspecific antagonist ICI 182,780 (ICI; 100 nmol/L). HUVECs were pre-treated for 30min with antagonist, followed by 30 min treatment with agonists and plated at a density of 4000 cells/ 50 µl per well on a matrigel–coated 15-well µ- slides. After incubation overnight the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=6 *P<0.05 relative to control, §P<0.05 relative to G1 or E2 treated HUVECs, using ANOVA test.

Similar results were obtained with the sprouting assay, where we observed an upregulation of sprout number per bead by G1 and E2 from 6.9±0.62 to 12.2±0.5 and 9.33±1, respectively ($p<0.05$ relative to control; Figure 15A). Co-treatment with G15 or ICI inhibited G1-stimulated sprout number from 12.2±0.5 to 5.9±0.31 and 6.2±0.41, respectively ($p<0.05$ relative to G1 treated HUVECs; Figure 15B).

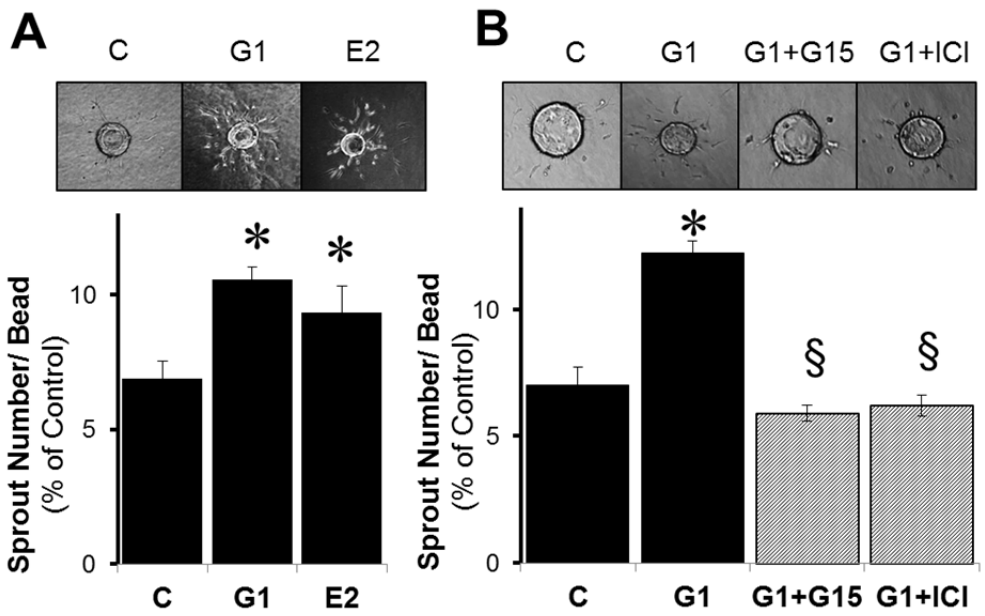

Figure 15. Bar graphs and representative photomicrographs showing the effects on sprout formation. HUVEC-coated Cytodex3 beads were plated in fibrin gels and cultured in DMEMF12 medium. They were treated with (**Panel A),** GPER specific agonist (G1; 10 nmol/L) or estradiol (E2; 10nmol/L) alone or in the presence of (**Panel B),** GPER specific antagonist (G15; 100 nmol/L) or ER-unspecific antagonist ICI 182,780 (ICI; 100 nmol/L). Cells were pretreated with antagonists for 30 min, subsequently G1 was added. After two days of incubation gels were photographed and the sprouts counted using Excellence Pro software. Values represent mean±SEM, n=5, *P<0.05 relative to control, §P<0.05 relative to G1 treated HUVECs, using ANOVA test.

The role of GPER in regulating endothelial function was further confirmed using Pertussis Toxin (PTX), an unspecific G-protein inhibitor. At a concentration of 0.1 ng/ml, PTX significantly reduced the stimulatory effects of G1 and E2 (both 10 nmol/L) from 210±41% to 20±9.9% and from 517±46% to 15±8%, respectively ($p<0.05$ relative to G1 or E2 treated HUVECs; Figure 16A).

Next, we silenced the expression of GPER in HUVECs, by transfecting them with GPER-specific siRNA (50 nmol/L). As shown in Figure 16B, GPER protein expression was significantly reduced by approximately 77% ($p<0.05$ compared to scrambled control). The silencing of GPER abrogated the capillary stimulating effects of G1 and E2. As shown in Figure 16C, capillary formation by G1 (10 nmol/L) and E2 (10 nmol/L) changed from 186±17% in GPER silenced control to 182±12% and 198±21% not significant, respectively. In contrast to HUVECs, treated with scrambled siRNA, G1 and E2 still induced capillary formation from 100% to 159±10% and 146±8%, respectively ($p<0.05$ relative to scrambled control, Figure 16C). These findings suggest that stimulation of the capillary formation in HUVECs is GPER

specific, and thereby might induce the beneficial effects of estrogens in cardiovascular repair process.

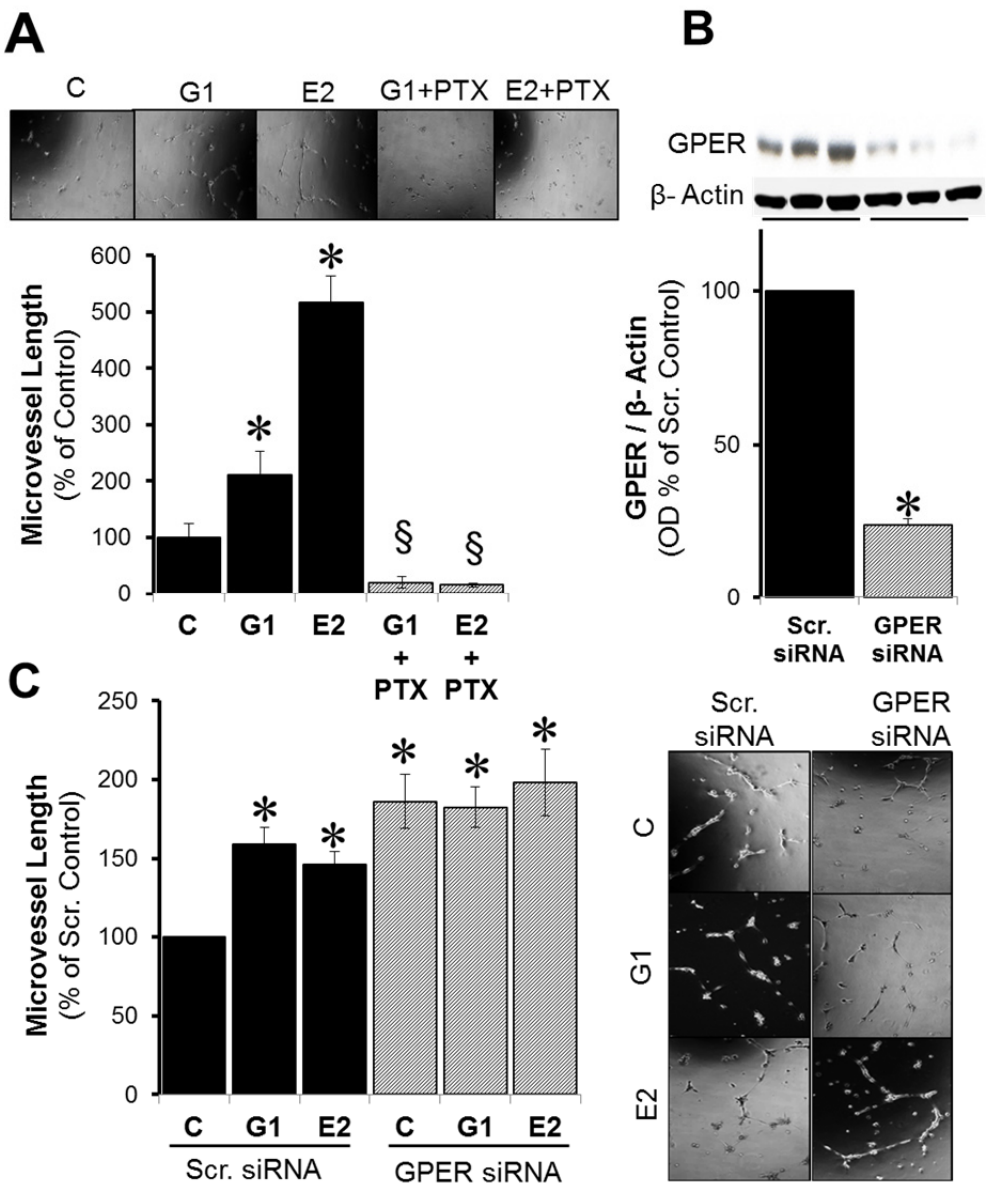

Figure 16. Bar graph and representative photomicrographs showing the effects of **Panel A,** GPER specific agonist (G1; 10 nmol/L), estradiol (E2; 10 nmol/L) and Pertussis Toxin (PTX; 0.1ng/ ml) on the capillary formation by HUVECs. Cells were plated at a density of 4000 cells/ 50 µl per well on a matrigel–coated 15-well µ- slides and pretreated with PTX for 30 min, subsequently G1 or E2 was added. After incubation overnight, the capillary formation was assessed microscopically (see Method section 3.2.2). The values represent mean±SEM, n=4, *P<0.05 relative to control, §P<0.05 relative to G1/ E2 using ANOVA test using ANOVA test. **Panel B,** Bar graph and representative Western Blot, showing the efficacy of silencing GPER in HUVECs using the transfection with scrambled siRNA (50 nmol/L) and GPER siRNA (50 nmol/L). Values represent mean±SEM, n=3; *P<0.05 relative to scrambled control using ANOVA test. **Panel C,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L), and estradiol (E2; 10 nmol/L) on capillary formation by HUVECs, transfected with scrambled siRNA (50 nmol/L) or GPER siRNA (50 nmol/L). HUVECs were plated at a density of 4000 cells/ 50 µl per well on a matrigel–coated 15-well µ- slides. After incubation overnight the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=3, *P<0.05 relative to scrambled control using ANOVA test.

4.1.2 GPER Activation induces Migration and Proliferation

Endothelial damage due to mechanical force or inflammation contributes to the onset for the initiation/ progression of CVD. Therefore, increased migration and proliferation of endothelial cells to repair and reestablish an intact endothelium is essential for preventing vaso-occlusive disorders.

To investigate the effects of GPER activation on migration/ wound closure, we performed scratch assays. Monolayers of HUVECs were “wounded” and treated with 10 nmol/L G1 or 10 nmol/L E2. After 24 hours we observed accelerated wound closure from 100% to 145±11% and to 144±6.6% in G1 and E2 treated cells, respectively ($p<0.05$ relative to control; Figure 17A). Moreover, G1-induced wound closure was significantly reduced by G15 (100 nmol/L) and ICI 182,780 (100 nmol/L) from 145±11% to 93±9.4% and 79±9.3%, respectively ($p<0.05$ relative G1 treated HUVECs; Figure 17B).

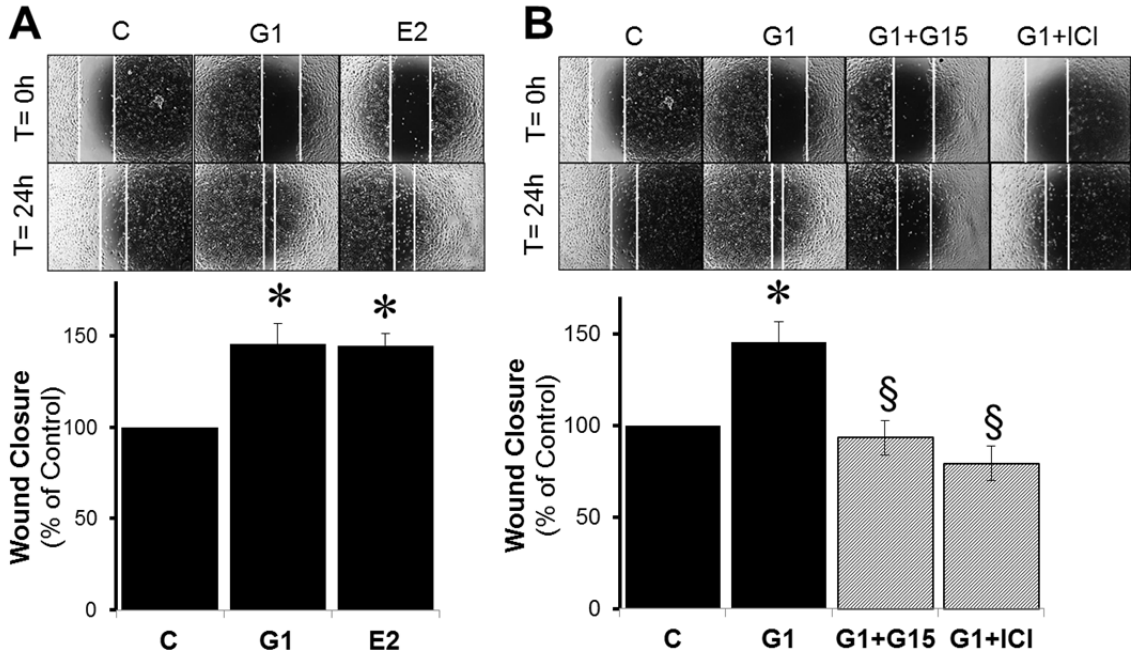

Figure 17. Bar graphs and representative photomicrographs showing the effects on wound closure. After scratching a confluent monolayer of HUVECs, wound closure was monitored following treatment with **Panel A**, GPER specific agonist (G1; 10 nmol/L), estradiol (E2; 10 nmol/L), **Panel B**, GPER specific antagonist (G15; 100 nmol/L) and ER-unspecific antagonist ICI 182,780 (ICI; 100 nmol/L). Cells were pretreated with antagonists G15 and ICI for 30 min and subsequently G1 was added for 24 h. Pictures were taken at timepoint 0 and 24 h afterwards and analysed using Excellence Pro software. Shown are mean±SEM, n=4, *P<0.05 relative to control, §P<0.05 relative to G1 treated HUVECs, using ANOVA test.

The impact of GPER activation on proliferation of endothelial cells was assessed by counting the cell number. In HUVECs treated for four days with G1 (10 nmol/L), cell proliferation was induced from 100±2.2% to 117±6.6% ($p<0.05$ relative to control). This increasing cell proliferation was suppressed by G15 (100 nmol/L) to 77±3.2% ($p<0.05$ relative G1 treated HUVECs; Figure 18). These observations suggest an important mediator role of GPER in migration and proliferation of HUVECs, which may underlie the beneficial effects of estrogens in cardiovascular repair process.

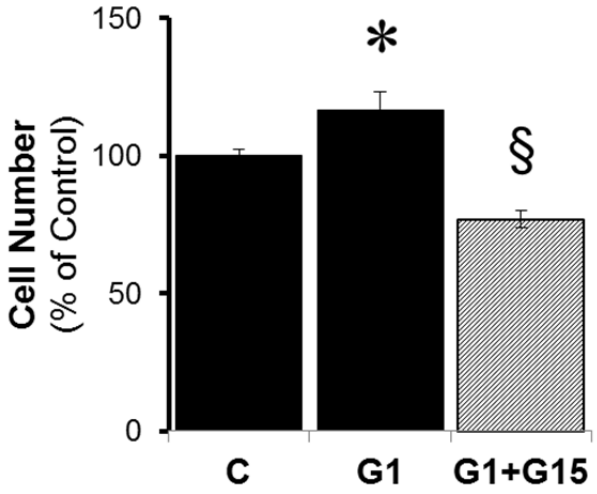

Figure 18. Bar graph showing the effects of a GPER specific agonist (G1; 10 nmol/L) or GPER specific antagonist (G15; 100 nmol/L) on proliferation of HUVECs. Cells (20,000cells/ well) were plated in 12-well plates and fed with complete media. After 48 h cells were starved overnight and pretreated with GPER antagonist G15 for 30 min and subsequently with G1. Treatment was renewed every 2 days and the experiments stopped after 4 days and cell number counted, using the Coulter Counter. Shown are mean±SEM, n=3, *$P<0.05$ relative to control, §$P<0.05$ relative to G1 treated HUVECs, using ANOVA test.

Discussion

The role of estrogens in the cardiovascular system is well established; however the mechanisms involved remain unclear.

Estrogen stimulates endothelial function by activating ERα and/ or ERβ [78, 102]. Recently, a third estrogen receptor, GPER has been identified [38], which seems to have an important regulatory role within the cardiovascular system. GPER is expressed within the vasculature [248], intact arteries [112] and ECs [25, 249].

Hence, using the HUVECs as a model, we assessed the role of GPER activation in regulating EC function. The development of GPER specific agonist G1 in 2006 [45] and GPER specific antagonist G15 in 2009 [46] facilitated our investigation to distinguish the estrogenic effects, solely elicited via GPER. Here we studied the effects of the GPER specific agonist G1 and of E2 on capillary formation, using two different *in vitro* models.

In the matrigel-based vasculogenesis assay G1 and E2 significantly stimulated microvessel formation by HUVECs. These observations are consistent with the

findings of Baruscotti et al. and De Francesco et al. [100, 250]. Because the stimulatory effect of E2 was 2.5 fold higher than the effect of G1, we speculate that a possible crosstalk of GPER and ERα, as recently suggested in BG-1 ovarian cancer and uterine epithelial cells [251, 252], might be responsible for this additive effect. The stimulatory effects on capillary formation were abolished by the GPER specific inhibitor G15 and the ER unspecific inhibitor ICI 182,780, further supporting our hypothesis for a GPER- ERα interaction. Surprisingly, other groups have observed that ICI 182,780 acts like an agonist for GPER and induces GPER signaling in breast cancer cell lines [40, 253]. However, in our HUVEC model we observed significant inhibition of the G1-stimulated effects with ICI 182,780. A possible explanation for these contraindicative observations might be the use of different cell lines. To further elucidate the stimulatory role of GPER in capillary formation, we applied the broad spectrum GPCR inhibitor Pertussis Toxin (PTX), which inactivates the GPCR subunits Gαi and Gαo [254]. While PTX alone induced angiogenesis of murine brain ECs [255], studies showed that PTX blocked GPER induced proliferation of OVCAR5 cells [256] and Akt activation in HEC-1A cells [257]. In line with these observations, we were able to block the stimulatory effects of G1 and E2 on capillary formation by HUVECs with PTX. This result suggests that the stimulatory effects of G1 and E2 on capillary formation are indeed mediated via classical GPCR mechanisms. To further confirm this notion, we silenced GPER in HUVECs by transfecting them with GPER specific siRNA. Silencing of GPER abolished the stimulatory effects of G1 and E2 on capillary formation. In contrast, G1 and E2 significantly induced microvessel formation in HUVECs transfected with scrambled siRNA. However, compared to cells treated with scrambled control, treatment with GPER silenced control surprisingly exhibited a significant increase in capillary formation. Other studies using GPER siRNA observed a significant decrease in G1-induced proliferation of CAFS [258] and proliferation, migration and invasion of SKOV3 cells [259] upon silencing of GPER. A possible explanation for these contrasting outcomes could be again the use of different cell lines. The use of different GPER siRNAs can be excluded as a pooled GPER siRNA was used within this present study.

GPER is a typical GPCR and as such coupled to heterotrimeric G-proteins Gα/ Gβ/ Gγ, each leading to specific signaling. Different subtypes exist from these heterotrimeric G-proteins, for GPER mediated signaling the subtypes Gαs [41] and Gβγ [38] have been suggested. We propose that upon silencing GPER, still unknown

pro-angiogenic or pro-survivor subunits of GPER may have been exposed, thereby contributing to this increased capillary formation in HUVECs. Nevertheless, upon silencing GPER, the stimulatory effects of G1 and E2 were lost, indicating the importance of GPER`s role in mediating estrogenic effects on capillary formation in HUVECs.

To form new blood vessels, ECs have to differentiate into tip and stalk cells with increased proliferative and migratory properties, leading to sprouting of ECs [260]. Therefore, we investigated the effects of G1 and E2 on sprouting of HUVECs using the Cytodex Bead assay. We observed increased sprouting in the presence of G1 and E2, which was significantly blocked by G15 and ICI 182,780. Taken together, our results provide evidence that GPER activation exerts stimulatory effects on vasculogenesis by HUVECs, by inducing microvessel/ capillary formation and sprouting.

Following endothelial denudation rapid repair of vascular wounds by increased migration and proliferation of ECs contributes to the pro-angiogenic and vasoprotective effects of E2 [102, 245]. To study the migration of HUVECs, we used the *in vitro* scratch assay method, where a monolayer of cells is “wounded” and the area of wound closure assessed, as previously described [261]. Similar to the effects of E2, we observed a significant increase in wound closure activity of HUVECs upon treatment with G1. The “wound closure” effects of G1 were abolished in presence of G15 and ICI 182,780, suggesting that GPER plays an important role in mediating the stimulatory effects of estrogens EC migration. Additionally, we assessed the effect of G1 on HUVECs proliferation and found a significant increase in cell number in response to G1, supporting its pro-mitogenic actions. Since we could block this increase in proliferation with G15, this indicates a high specificity of GPER in inducing HUVECs proliferation. Together, these results provide evidence that GPER stimulated migration and proliferation potentially contribute to its pro-vasculogenic effects in the HUVECs.

Many studies have observed positive effect of estrogen on proliferation and migration of endothelial cells in vitro [94], promotion of endothelial regrowth after arterial injury [95] and acceleration of re-endothelialization [96]. Additionally, mobilization of ECFCs, inflammatory immune cells and platelets by E2 might also contribute to the repair of the endothelium [97, 98]. Moreover, E2 stimulates angiogenesis in vitro and

in vivo [99-101] by inducing the most important pro-angiogenic factors: Fibroblast growth factor 2 (FGF2), VEGF and NO [99].

In contrast to our findings, some studies have shown that activation of GPER inhibits EC proliferation [38, 249], thus suggesting an anti-angiogenic effect. However, the positive effect of GPER in the cardiovascular system is indirectly supported by the fact that: 1) infusion with GPER specific agonist G1 lowers blood pressure in normotensive [114] and hypertensive rats [108]; 2) G1 treatment improves diastolic dysfunction, cardiac hypertrophy and decreases myocyte size [129]; and 3) GPER induces vasodilatory action [108, 113, 114] in a NO-dependent fashion [113, 114, 117]. Apart from its vasodilatory effect, NO also stimulates angiogenesis [192] and therefore could be responsible for GPER`s stimulatory effect on capillary formation in our HUVEC model.

With regard to our findings in HUVECs, we postulate that stimulation of GPER induces endothelial function by promoting proliferation, migration and angiogenesis in ECs. In summary, GPER might play an important role in mediating the protective effects of estrogen on the cardiovascular system. GPER specific agonist G1 may be of therapeutic and clinical relevance in targeting vasoocclusive disorders. Since G1 is non-feminizing, it could be of therapeutic interest in both men and women.

4.2 The ALK1/ SMAD1/5/8 Pathway mediates GPER-induced Capillary Formation in Endothelial and Progenitor Cells

Objective

Our findings from capillary formation experiments demonstrated that the pro-vasculogenic effects of estradiol are mimicked by G1, a GPER specific agonist in HUVECs. Hence, we further investigated the potential underlying mechanisms. Since ALK1/ SMAD1/5/8 signaling pathway plays an important role in angiogenesis, we investigated its role in mediating GPER-induced vasculogenesis in HUVECs and ECFCs.

Introduction

The superfamily of TGFβ proteins includes TGFβ, the BMP subfamily, activins, and GDFs. The different ligands and their downstream pathway components are highly conserved and regulate multiple diverse cellular functions such as growth, adhesion, migration, apoptosis and differentiation [142]. Members of the TGFβ family dimerize and activate heteromeric complexes of type I and type II transmembrane receptors, leading to an activation of the downstream transcription factors SMADs [262, 263]. Depending on the cell type and the context, diverse responses can be triggered upon binding of the same member of the TGFβ family by recruiting a distinct set of receptors [264]. ECs highly express ALK1, which is a type I transmembrane receptor and activates SMAD1/5/8 [164]. Research suggests a key role of this ALK1/ SMAD1/5/8/ signaling in embryonic angiogenesis, since mutations in *ALK1* and *SMADs* lead to severe defects in embryogenesis and early lethality [176-178]. The ALK1/ SMAD1/5/8 pathway might also play a role in maintaining homeostasis of the vasculature, as patients with HHT, suffering from telangiectasis, arteriovenous malformations, nose bleeds and gastrointestinal bleeding, show mutations in *ALK1* [144].

In summary, the impact of ALK1 signaling in ECs, vasculogensis and vascular integrity is evident. In the present study, we investigated whether activation of GPER modulates ALK1/ SMAD1/5/8 signaling in HUVECs and whether this pathway contributes to G1-induced vasculogenesis.

Methods

As described in section 3.2.

4.2.1 Activation of ALK1/ SMAD1/5/8 Pathway

The ALK1/ SMAD1/5/8-pathway is known to be pro-angiogenic and, as shown in Figure 19A, treatment of HUVECs with G1 (100 nmol/l) induced ALK1 and ID-1 expression by more than 1.5 fold (from 100±4.5% to 168±21.2% and from 100±9.7% to 179±26.2%, respectively; $p<0.05$ relative to control), while the expression of ALK5, which opposes ALK1 actions, was decreased and the expression of PAI-1, which is a downstream target of ALK5, was not altered (from 100±4.2% to 63±8.5% and from 100±1% to 103±4.67%, respectively; $p<0.05$ relative to control; Figure 19B).

Activation of ALK1 leads to phosphorylation of SMAD1/5/8. Since we observed an upregulation of ALK1 expression by G1, we further studied changes in SMAD1/5/8 phosphorylation following GPER activation. We observed increased phosphorylation of SMAD1/5/8 from 100% to 208±36% ($p<0.05$ relative to control, Figure 20A) upon treatment of HUVECs with G1 (10 nmol/ L) for 45 min. Pre-treatment with G15 (100 nmol/L), a GPER specific antagonist, or ICI 182,780 (100 nmol/L), a non-specific ER antagonist, reduced the G1-stimulated phosphorylation of SMAD1/5/8 from 208±36% to 117±23.7% and 131±27.8%, respectively ($p<0.05$ relative to G1 treated HUVECs; Figure 20A).

The regulatory role of GPER on SMAD1/5/8 phosphorylation was further confirmed in HUVECs, where GPER was silenced. Indeed, treatment with G1 (10 nmol/L) and E2 (10 nmol/L) had no effect on the phosphorylation of SMAD1/5/8 (from 74±4.5% to 76±12.2% and 36±27.7%, respectively) as compared to GPER silenced controls, while both G1 and E2 induced the phosphorylation of SMAD1/5/8 in HUVECs treated with scrambled siRNA from 100% to 135±5.5% and 131±2.3%, respectively ($p<0.05$ relative to scrambled control, Figure 20B).

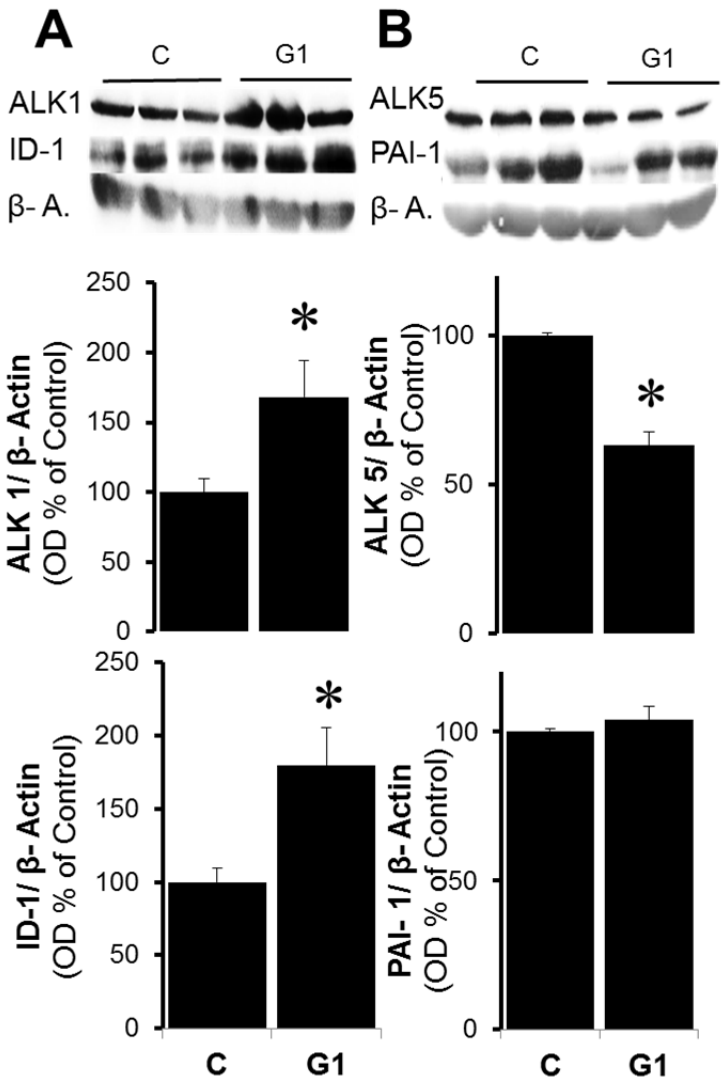

Figure 19. Bar graph and representative Western Blots demonstrating the effects of treatment with GPER specific agonist (G1, 100 nmol/L) on protein expression of ALK1, ID-1 **(Panel A)** and of ALK5 and PAI-1 **(Panel B)**. HUVECs were starved ON and treated for 24h. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test.

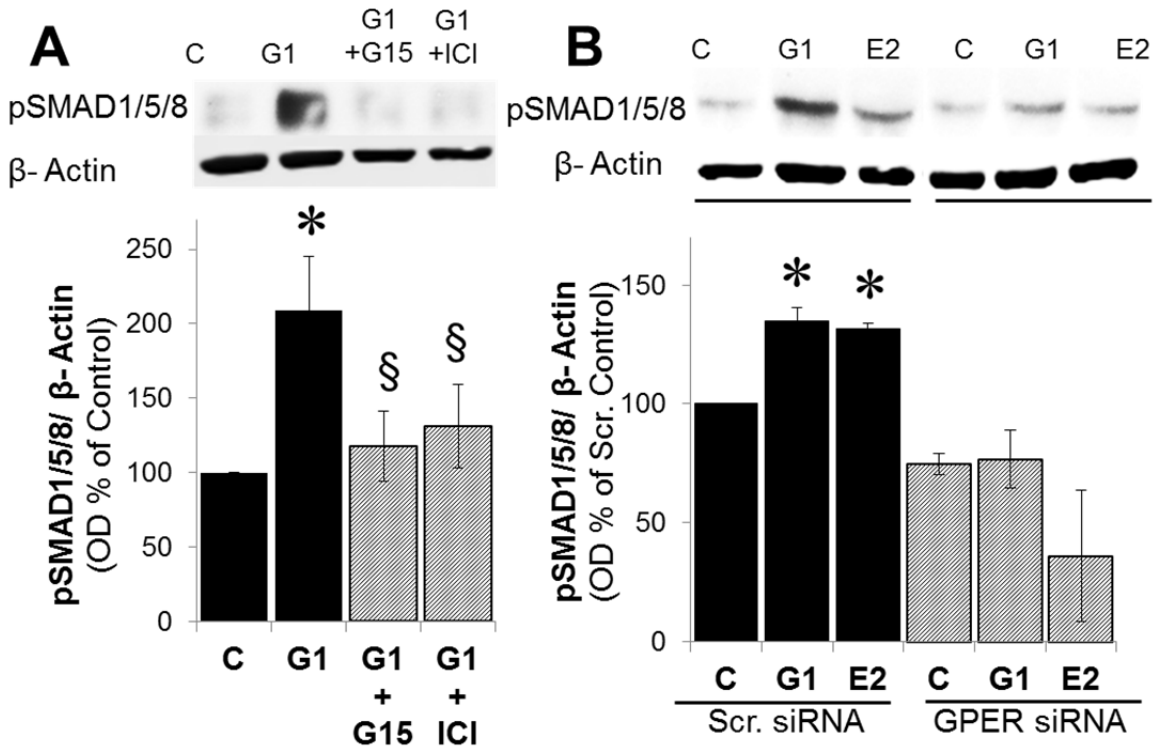

Figure 20. Bar graphs and representative Western Blots demonstrating the effects of GPER activation on SMAD1/5/8 phosphorylation. **Panel A,** depicts the effects of GPER specific agonist (G1; 10 nmol/L), GPER specific antagonist (G15; 100nmol/L) and ER-unspecific antagonist ICI 182,780 (ICI; 100 nmol/L) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs were treated with antagonists for 30min, subsequently G1 was added for 45min. Values represent mean±SEM, n=5, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test. **Panel B,** depicts effects of GPER specific agonist (G1; 10 nmol/L) and estradiol (E2; 10 nmol/L) on the phosphorylation of SMAD1/5/8 in pre-starved HUVECs, transfected with scrambled siRNA (50 nmol/L) or GPER siRNA (50 nmol/L). Values represent mean±SEM, n=3, *P<0.05 relative to scrambled control, using ANOVA test.

Recent studies provide evidence that circulating progenitor ECs are capable of repairing the damaged endothelium and form capillaries in response to E2. To confirm the regulatory role of GPER on the ALK1/ SMAD1/5/8 pathway and the impact on capillary formation in progenitor ECs, we investigated this pathway also in ECFS. As shown in Figure 21A, treatment of ECFCs with G1 (100 nmol/L) induced ALK1 and ID-1 expression by 2-fold (from 100±9.8% to 202±24.8% and from 100±5.4% to 207±24.8%, respectively; $p<0.05$ relative to control). In contrast to ALK1 and ID-1, G1 attenuated ALK5 and PAI-1 expression from 100±12.4% to 85±9.2% and from 100±10% to 76±13.96%, respectively ($p>0.05$ relative to control; Figure 21B). The phosphorylation of SMAD1/5/8 was increased in ECFCs treated with G1 (10 nmol/L) from 100% to 212±3% ($p<0.05$ relative to control), and this was significantly reduced upon pre-treatment with G15 (100 nmol/L) and ICI 182-780 (100 nmol/L) to 120±11% and 101±5.8%, respectively ($p<0.05$ relative to G1 treated

ECFCs; Figure 21C). These results in ECFCs also reflect and support our data obtained in HUVECs, suggesting that GPER activation induces the ALK1/ SMAD1/5/8 pathway in both HUVECS and ECFCs.

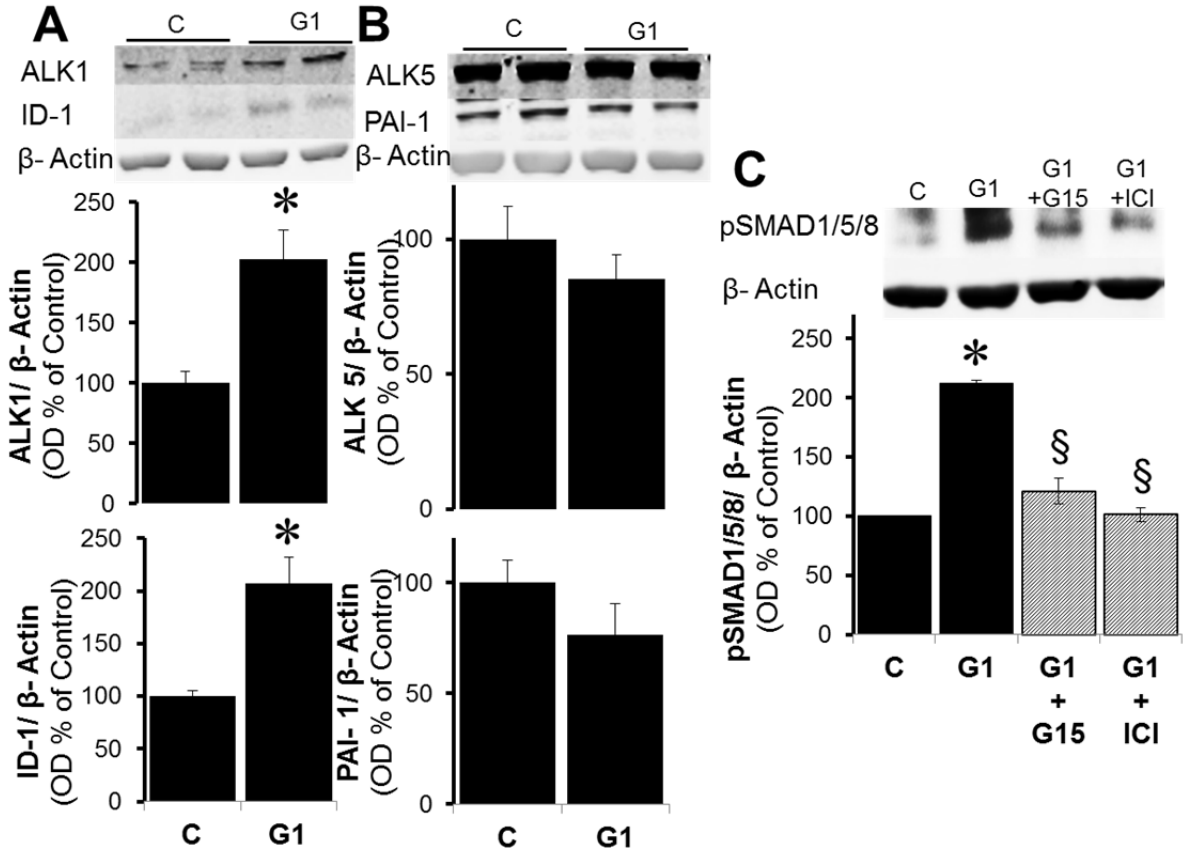

Figure 21. Bar graphs and representative Western Blots demonstrating the effects of the GPER specific agonist (G1, 100 nmol/L) in ECFCs treated for 24 h on protein expression of **(Panel A)**, ALK 1 and ID-1, and **(Panel B),** ALK 5 and PAI-1. **Panel C** shows the phosphorylation of SMAD1/5/8 in pre-starved ECFCs. The cells were treated with the GPER specific antagonist (G15; 100nmol/L) or the ER-unspecific antagonist ICI 182,780 (ICI; 100 nmol/L) for 30min. Subsequently, G1 (10 nmol/L) was added for 45 min. Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

4.2.2 Role of ALK1/ SMAD1/5/8 Pathway in GPER-induced Capillary Formation

Our results suggest that GPER activation induces the ALK1/ SMAD1/5/8 pathway, which is known to induce capillary formation in endothelial cells. Therefore, we studied the role of G1- induced SMAD1/5/8 signaling in mediating vasculogenesis in ECs.

For this purpose, we applied ALK1Fc (100 ng/ml), which is a specific antagonizing antibody and pharmacological inhibitor for ALK1, and SJN2511 (SJN, 100 nmol/L), a specific antagonist of ALK5, and examined their impact on the G1-induced phosphorylation of SMAD1/5/8. As shown in Figure 22A, treatment with G1 induced

SMAD1/5/8 phosphorylation and this effect was significantly abrogated by ALK1Fc from 189±26% to 24±8.9%, whereas pre-treatment with SJN had no modulatory effect and altered SMAD1/5/8 phosphorylation from 189±26% to 166±45.6%, ($p<0.05$ relative to G1 treated HUVECs).

Next we applied the same antagonists prior to G1 treatment in HUVECs and assessed microvessel formation. ALK1Fc (100 ng/ml) significantly blocked G1-induced capillary formation by 94% ($p<0.05$ relative to G1 treated HUVECs), but SJN (100 nmol/L) was ineffective and did not alter capillary formation, which was 156±8.2% to 159±19.5% respectively, in the absence or presence of SJN ($p>0.05$ relative to G1 treated HUVECs; Figure 22B).

The importance of the ALK1/ SMAD1/5/8 pathway in GPER mediated capillary formation was further confirmed by silencing SMAD1 in HUVECs transfected with SMAD1 specific siRNA. High silencing efficacy was obtained, as SMAD1/5/8 expression was decreased by 41% and SMAD1/5/8 phosphorylation following BMP9 treatment (10 ng/ml) was reduced by approximately 70% ($p<0.05$ relative to scrambled control; Figure 23A). In capillary formation experiments, silencing of SMAD1 abrogated the stimulatory effects of G1 (10 nmol/L) and E2 (10 nmol/L), which changed from 77±5.3% to 94±6.8% and 92±10.2% ($p>0.05$ relative to SMAD1 silenced control). In contrast, in HUVECs, treated with scrambled siRNA, G1 and E2 induced capillary formation from 100% to 159±10% and 146±8%, respectively ($p<0.05$ relative to scrambled control, Figure 23B). These data suggest that GPER-induced vasculogenesis in ECs is mediated via the ALK1/SMAD1/5/8 pathway and confirms the importance of this pathway in capillary formation.

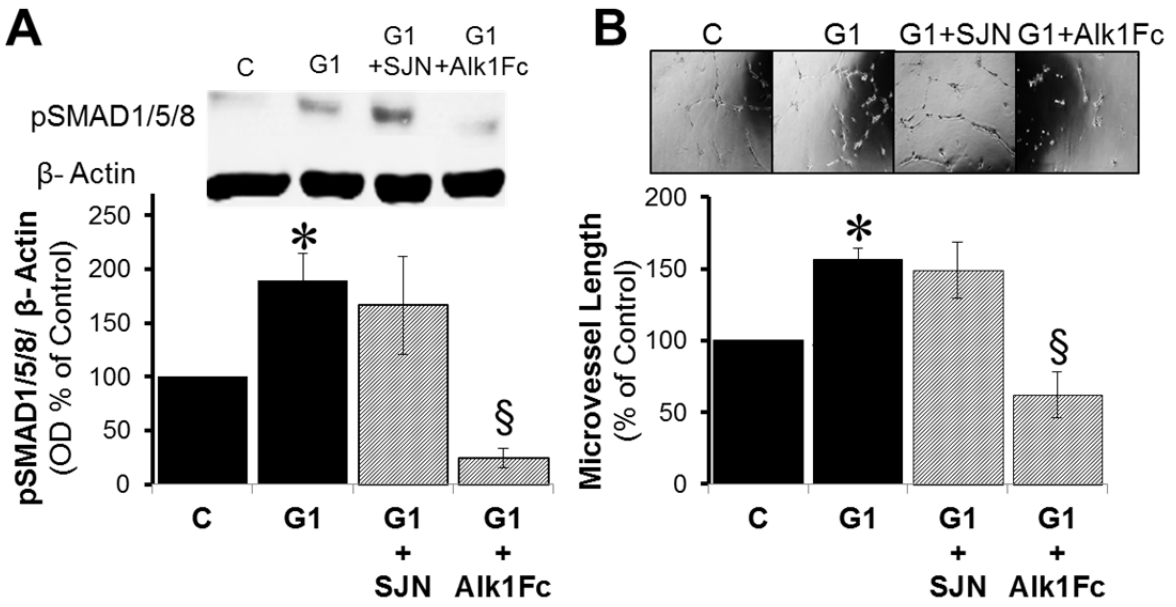

Figure 22. Panel A, Bar graph and representative Western Blot demonstrating the effects of GPER specific agonist (G1; 10 nmol/l), ALK5 specific antagonist (SJN; 100 nmol/L) and ALK1 specific antagonizing antibody (Alk1Fc; 100 ng/ml) on the phosphorylation of SMAD1/5/8. HUVECs were starved for 4 h and treated with the antagonists for 30 min, G1 was then added for 45 min. Values represent mean±SEM, n=6, *P<0.05 relative to control, §P<0.05 relative to G1, using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L), ALK 5 specific antagonist (SJN; 100 nmol/L) and ALK1 specific antagonizing antibody (Alk1Fc; 100 ng/ml) on capillary formation by HUVECs. The cells were plated at a density of 4000 cells per well on a matrigel–coated 15-well µ-slides and pretreated with the antagonists for 30 min, subsequently G1 was added. After overnight incubation, the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=6, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

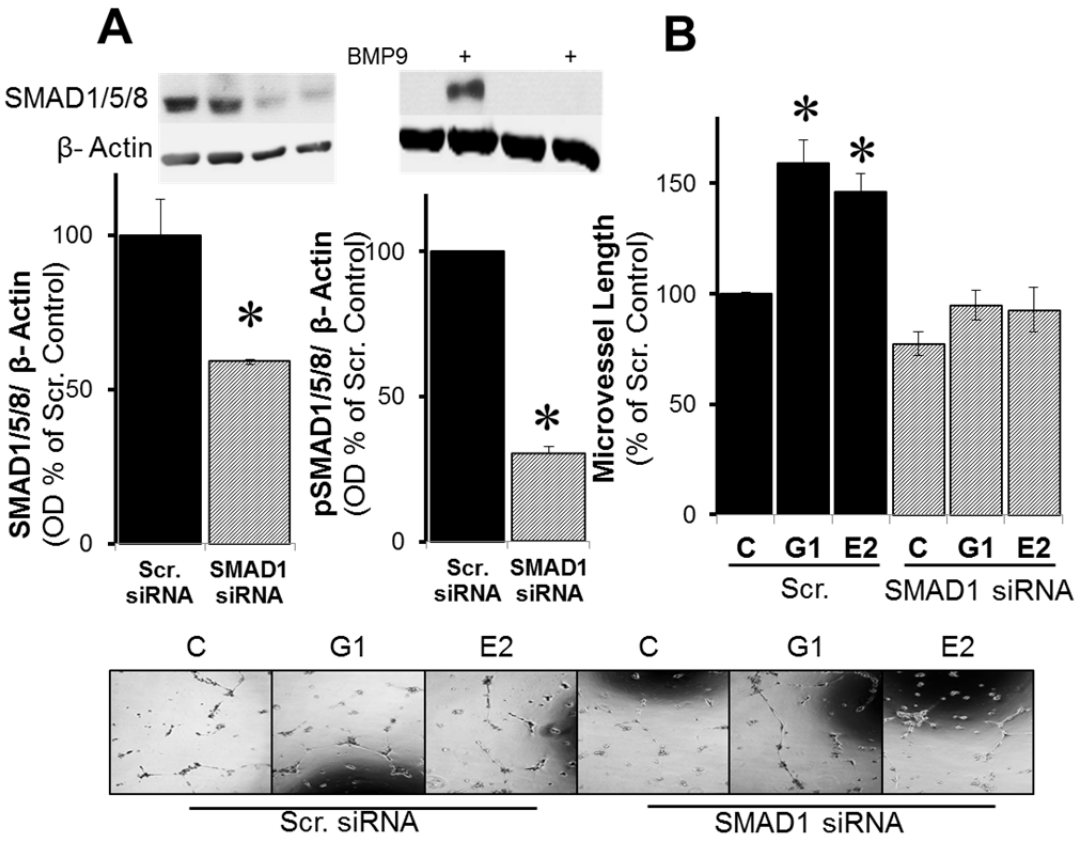

Figure 23. Panel A, Bar graph and representative Western Blot showing the efficacy of transfection with scrambled siRNA (50 nmol/L) and SMAD1 siRNA (50 nmol/L) in HUVECs. Values represent mean±SEM, n=4, *P<0.05 relative to scrambled control using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L), and estradiol (E2; 10 nmol/L) on capillary formation by HUVECs transfected with scrambled siRNA (50 nmol/L) or SMAD1 siRNA (50 nmol/L). HUVECs were plated at a density of 4000 cells per well on a matrigel–coated 15-well μ- slides. After overnight incubation the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=3, *P<0.05 relative to scrambled control using ANOVA test.

We also investigated the impact of G1- induced ALK1/ SMAD1/5/8 signaling in mediating vasculogenesis by ECFCs. Similar to our observations in HUVECs, treatment of ECFCs with ALK1Fc (100 ng/ml) abrogated G1-induced SMAD1/5/8 phosphorylation from 205±19% to 118±15.2. ($p<0.05$ relative to G1 treated ECFCs; Figure 24A). Moreover, treatment with G1 (10 nmol/L) induced capillary formation from 100% to 135±10.7% ($p<0.05$ relative to control) and pre-treatment with ALK1Fc significantly blocked G1-induced capillary formation from 135±10.7% to 58±17% ($p<0.05$ relative to G1 treated ECFCs; Figure 24B). In summary, our results from the ECFCs experiments confirm our observations in HUVECs and suggest that GPER induced- vasculogenesis is mediated via activation of the ALK1/ SMAD1/5/8 pathway.

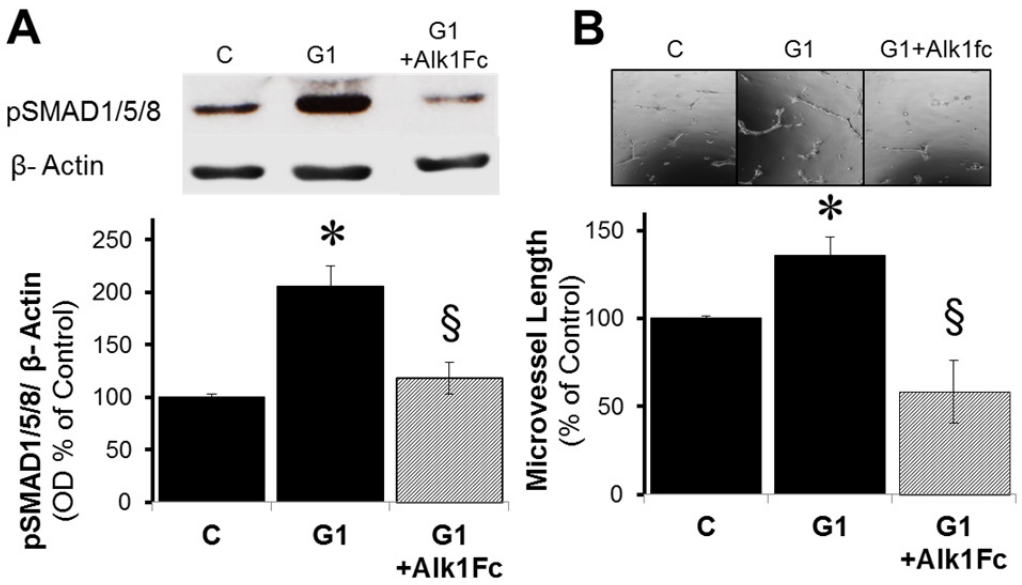

Figure 24. Panel A, Bar graph and representative Western Blot demonstrating the effects of **A,** GPER specific agonist (G1; 10 nmol/l) and ALK 1 specific antagonizing antibody (Alk1Fc; 100 ng/ml) on the phosphorylation of SMAD1/5/8. Pre-starved ECFCs were pretreated with the antagonists for 30min, subsequently G1 was added for 45min. Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L) and ALK 1 specific antagonizing antibody (Alk1Fc; 100 ng/ml) on capillary formation by ECFCs. The cells were plated at a density of 4000 cells per well on a matrigel–coated 15-well µ- slides and pretreated with the antagonists for 30min. G1 was then added for incubation overnight and capillary formation assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

4.2.3 GPER-induced Capillary Formation and SMAD1/5/8 Phosphorylation involves BMP2

BMP2 is an endogenous ligand for BMP receptor type I [265], which leads to dimerization of TGFβ receptor I (e.g. ALK1) with the type II receptor and receptor activation with subsequent activation of the signaling cascade. An endogenous counterplayer of BMP2 is Noggin, which blocks BMP2 and inhibits its binding to the BMP receptor type I [266]. Both molecules were employed to confirm our observations that the ALK1/ SMAD1/5/8 pathway plays an important and active role in the capillary formation of HUVECs. Indeed, treatment of HUVECs with BMP2 (10 ng/ml) induced the phosphorylation of SMAD1/5/8 from 100% to 322±43.1% and capillary formation from 100% to 133±4.2% ($p<0.05$ relative to control). Moreover, these effects of BMP2 were blocked significantly by Noggin (100 ng/ml) to 90±23.4% and to 109±13.5%, respectively ($p<0.05$ relative to BMP2 treated HUVECs; Figure 25A/ B). Next, we investigated whether Noggin affects G1 (10 nmol/L) -induced capillary formation and SMAD1/5/8 phosphorylation in HUVECs. Noggin blocked G1-

induced capillary formation from 141±7.1% to 103±9.4% and phosphorylation of SMAD1/5/8 from 190±25.8% to 28±9.4% ($p<0.05$ relative to G1 treated HUVECs; Figure 25C/ D). These findings led us to suggest that G1-induced capillary formation and SMAD1/5/8 phosphorylation potentially involves BMP2.

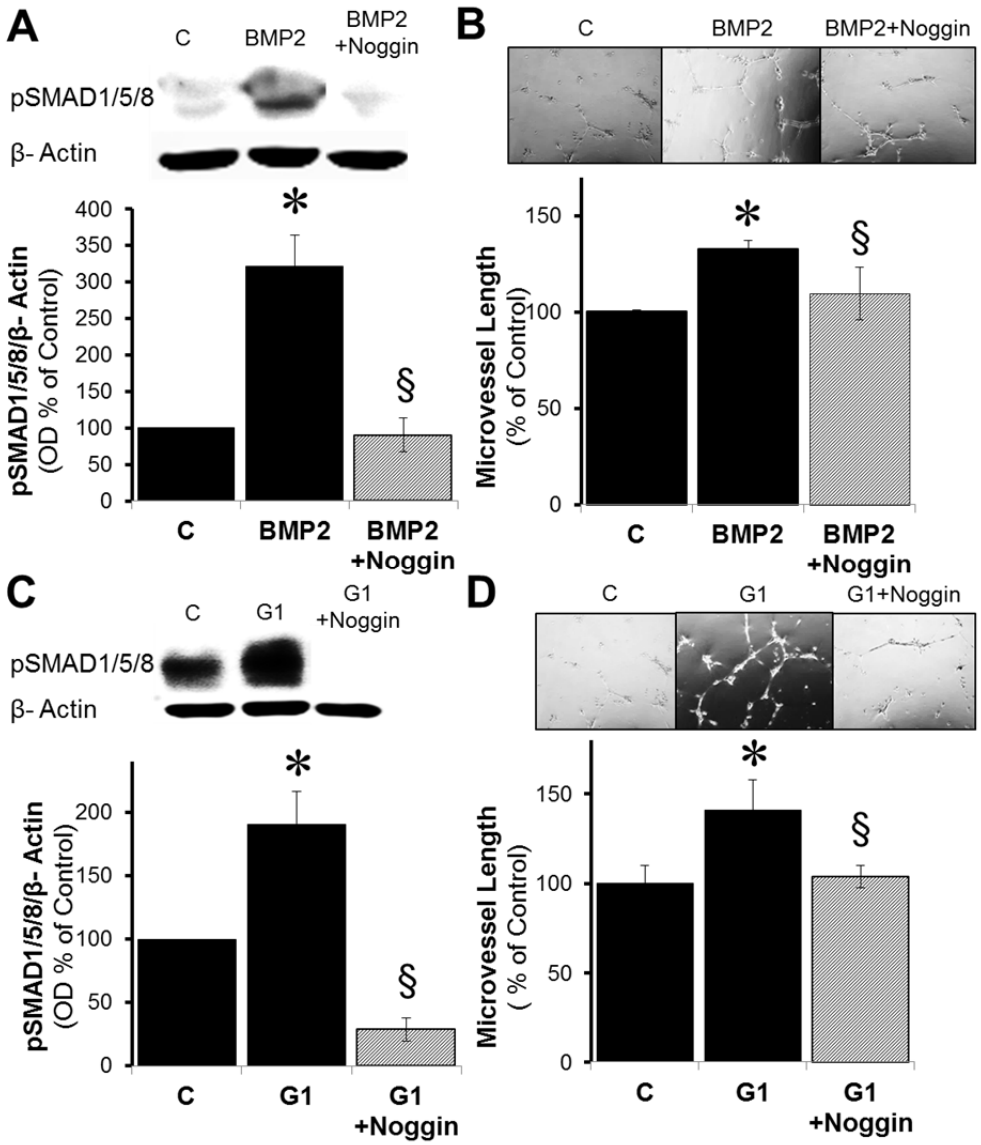

Figure 25. Panel A, Bar graph and representative Western Blot, demonstrating the effects of TGFβ Receptor II specific agonist (BMP2; 10 ng/ml), and BMP inhibitor (Noggin, 100 ng/ml) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs were treated with Noggin for 30 min, subsequently BMP2 was added for 45 min. Values represent mean±SEM, n=4, *P<0.05, relative to control, §P<0.05 relative to BMP2, using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of TGFβ Receptor II specific agonist (BMP2; 10ng/ml), and BMP inhibitor (Noggin, 100ng/ml) on capillary formation by HUVECs. Cells were plated in a density of 4000 cells per well on a matrigel–coated 15-well μ- slide and treated with the inhibitor for 30 min, subsequently BMP2 was added. After incubation overnight capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=4, *P<0.05 relative to control, §P<0.05 relative to BMP2 using ANOVA test. **Panel C,** Bar graph and representative Western Blot, demonstrating effects of GPER specific agonist (G1; 10 nmol/L) and BMP inhibitor (Noggin, 100ng/ml) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs were treated with inhibitor for 30 min, subsequently G1 was added for additional 45 min. Values represent mean±SEM, n=5, *P<0.05, relative to control, §P<0.05 relative to G1 using ANOVA test. **Panel D,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L) and BMP inhibitor (Noggin, 100ng/ml) on capillary formation by HUVECs. Cells were plated in a density of 4000 cells per well on a matrigel–coated 15-well μ- slide and treated with inhibitor for 30min, subsequently G1 was added. After incubation overnight capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=5, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

4.2.4 **Activation of BMP2 Secretion**

Our observation that Noggin blocks the effects of G1 on capillary formation and SMAD1/5/8 phosphorylation suggested a link between G1 and BMP2. Therefore, we investigated whether GPER activation results in the release of BMP2 from cultured cells into the supernatant. Indeed, analysis of the supernatant from G1 (10 nmol/L) - treated HUVECs revealed an increase in BMP2 level by 370±63.4% ($p<0.05$ relative to control) and this effect was significantly blocked by G15 (100 nmol/L) to 103±55.8% ($p<0.05$ relative to G1 treated HUVECs; Figure 26). Taken together our data provide evidence that G1 induces the phosphorylation of SMAD1/5/8 by triggering the release of BMP2 via a putative short term effect, which subsequently activates the signaling cascade leading to increased vasculogenesis.

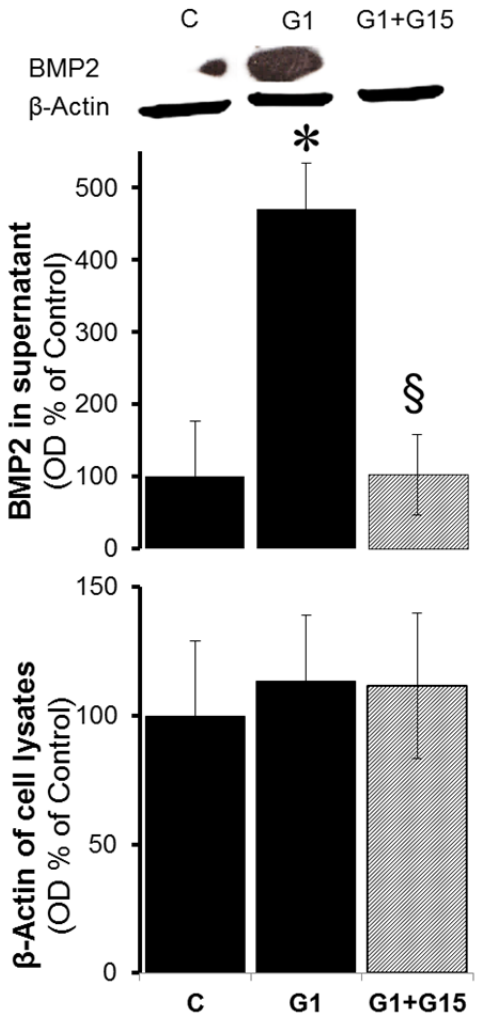

Figure 26. Bar graph and representative Western Blot, demonstrating the effects of GPER specific agonist (G1; 10 nmol/L) and GPER specific antagonist (G15; 100 nmol/l) on the release of BMP2 from HUVECS into the supernatant. For normalization, β-Actin was detected in whole cell lysates since it is not present in the supernatant. Pre-starved HUVECs were treated with the antagonist for 30 min in a volume of 200 µl. G1 was then added for 30 min. Values represent mean±SEM, n=3, *P<0.05, relative to control, §P<0.05 relative to G1, using ANOVA test.

Discussion

The ALK1/ SMAD1/5/8 signaling pathway is important in regulating endothelial cell function. It has been shown to be essential in human embryogenesis where it regulates angiogenic processes. Mutations in *ALK1* or *SMAD1/5/8* have been shown to cause early lethality due to vascular abnormalities in murine embryos [176-178]. Moreover, many studies demonstrated a prominent role of ALK1/ SMAD1/5/8 in modulating postnatal angiogenesis [160, 267-269].

ALK1 is mainly expressed in ECs [164, 270] and we found that GPER activation stimulated ALK1 expression in HUVECs and ECFCs. Moreover, G1 increased the expression of ID-1, the downstream target of SMAD1/5/8 activation, which is responsible for inducing migration and tube formation in ECs [153]. Interestingly, in both EC types we observed no change in ALK5 expression and its downstream target PAI-1 following G1 treatment. Activation of the ALK5- pathway, in contrast to the ALK1- pathway, leads to inhibition of EC proliferation, tube formation and migration [149, 151], thereby blocking angiogenesis.

Furthermore, we observed a significant increase in the phosphorylation of SMAD1/5/8 in HUVECs and ECFCs upon G1 treatment, further emphasizing an activation of this signaling pathway. Consistent with our observations, other studies already showed induction of SMAD1/5/8 phosphorylation by estradiol in mouse MC3T3-E1 cells and myoblastic C2C12 cells [271, 272].

In our study, SMAD1/5/8 phosphorylation was inhibited by GPER specific antagonist G15 in both HUVECS and ECFCs. Moreover, in HUVECs, transfected with GPER specific siRNA, the stimulatory effects of G1 and E2 on pSMAD1/5/8 were abolished. While Matsumoto et al. previously demonstrated the stimulatory effects of estrogens on SMAD1/5/8 phosphorylation [271, 272], to our knowledge, we are the first demonstrating the regulatory role of GPER in mediating the estrogenic effects on the phosphorylation of SMAD1/5/8.

In summary, the above findings suggest a potentially prominent role of GPER in activating ALK1/ SMAD1/5/8, but not ALK5, signaling.

Although the ALK1- and the ALK5- pathways elicit opposite responses, these two proteins are physically interacting and each is essential for maximal activation of the

other [151]. Based on this reported crosstalk, we used inhibitors for ALK1 and ALK5 to study their role in GPER-induced phosphorylation of SMAD1/5/8 and vasculogenesis in HUVECs and ECFCs.

We were able to block both G1-induced pSMAD1/5/8 and G1- stimulated capillary formation in both HUVECs and ECFCs with ALK1Fc, a specific neutralizing antibody for ALK1. These observations are consistent with other studies, which previously demonstrated a blocking effect of ALK1Fc on angiogenesis of HMVECs [174] and on the expression of ALK1`s downstream product ID-1 [149, 174, 273]. However, in HUVECs stimulated with G1, treatment the ALK5- inhibitor SJN251 had no reducing effects on the phosphorylation of SMAD1/5/8 and vasculogenesis. This is in line with the reported role of ALK5 in inhibiting angiogenesis [149, 151] and suggests that GPER-stimulated vasculogenesis and SMAD1/5/8 phosphorylation is mediated via ALK1, and that this is not dependent on crosstalk with ALK5 in our HUVEC model.

Recently, knocking out SMAD1 in endothelial and smooth muscle cells has been shown to be linked to Pulmonary Arterial Hypertension (PAH) [274], which is associated with abnormal angiogenesis [275]. Taking these results into account and to further elucidate the importance of ALK1/ SMAD1/5/8 in GPER- stimulated vasculogenesis, the HUVECs were transfected with SMAD1 specific siRNA. Indeed, silencing of SMAD1 completely abrogated the stimulatory effects of G1 and E2 on capillary formation of HUVECs.

In summary, this result emphasizes and reaffirms the essential role of ALK1/ SMAD1/5/8 signaling in mediating GPER-induced capillary formation by ECs.

Studies have shown that BMP2 and BMP4, belonging to the TGFβ superfamily, play important roles in endothelial cell function, by inducing differentiation, proliferation, migration and cell survival [276-278]. Consistent with other studies [279-281], we observed a significant increase in capillary formation of HUVECs upon BMP2 treatment. Moreover, the stimulatory effect of BMP2 on vasculogenesis was blocked by Noggin, an endogenous sequestering molecule, that inhibits the binding of BMP2 and BMP4 to their receptors and thereby blocks further signaling [266]. Moreover, the phosphorylation of SMAD1/5/8 was induced in HUVECs treated with BMP2 and significantly reduced upon pre-treatment with Noggin. BMP2 binds and activates the

BMP type I receptors ALK3 and ALK6 [265, 282], and phosphorylation of SMAD1/5/8 in response to BMP2 has already been observed and established in ECs [283].

Surprisingly, Noggin also inhibited G1-induced capillary formation and phosphorylation of SMAD1/5/8, which suggests a link between GPER and BMP2. Interestingly, BMP2 effects on differentiation of C2C12 cells could be significantly blocked with the GPCR-unspecific inhibitor PTX [284], suggesting that BMP2 functions are mediated via G-protein coupled mechanisms.

As described in the previous section 4.1, we were also able to block the effects of GPER, a classical GPCR, on vasculogenesis using PTX in HUVECs. This further supports the notion for a potential link between GPER activation and BMP2 signaling. Therefore to substantiate this, we investigated whether GPER activation induces the release of BMP2. Indeed, a significant increase in BMP2 was observed within the supernatant of HUVECs treated with G1. This significant effect could be blocked using G15, indicating high GPER specificity in stimulating BMP2 release.

Taken together, the above findings and our observation that GPER activation stimulates ALK1 expression and SMAD1/5/8 phosphorylation and leads to increased capillary formation, suggest that the vasculogenic effects of G1 are triggered/ initiated by G1-induced release of BMP2, via a putative short term effect, in HUVECs.

4.3 The Role of cAMP in GPER-induced Capillary Formation and ALK1/ SMAD1/5/8 Pathway in Endothelial Cells

Objective

Cyclic AMP plays an important role in mediating the biological effects of sex hormones. It is well established that the beneficial effects of estradiol on the cardiovascular system are in part mediated by cAMP. Indeed, stimulation of cAMP production induces endothelial function and inhibits SMC proliferation. Recently, it has been suggested that non-vascular and vascular cells generate cAMP upon GPER activation. Therefore we investigated whether GPER stimulates cAMP generation in HUVECs and whether cAMP signaling plays a role in GPER-stimulated ALK1/ SMAD1/5/8 pathway and vasculogenesis.

Introduction

It is well established that estrogen mediates rapid intracellular signaling events [233, 285-288], such as activation of adenylyl cyclase, leading to production of 3', 5'-cyclic adenosine monophosphate (cAMP). Cyclic AMP generation in response to estradiol was reported for the first time in rat uterus 1967 [234]. Subsequently, many other studies demonstrated that estrogen induces cAMP levels [104, 233-236], which triggers vasodilation [236, 289, 290] and inhibition of SMC proliferation [104, 291]. Keung et al. reported that ER-antagonist ICI 182,780 could not block estrogen-induced cAMP in vascular SMC [236] and suggested that stimulatory effects of estrogen on adenylyl cyclase might be mediated via a GPCR-dependent mechanism [292, 293]. The above studies suggest that the recently identified membrane GPER, a classical GPCR, might be responsible for the stimulatory effects of estradiol on cAMP. This notion was confirmed by several studies, which detected increased adenylyl cyclase activity and cAMP upon GPER activation in non-vascular cells [39, 41, 294] and vascular cells [295]. Furthermore, Amano et al. detected increased angiogenesis in *in vivo* models upon injection with 8-bromo-cAMP [191], indicating a pro-angiogenic mediator role of the cAMP signalling pathway.

Based on the above findings we hypothesized and assessed whether GPER activation stimulates cAMP production in HUVECs and whether cAMP signaling mediates GPER-induced vasculogenesis and ALK1/ SMAD1/5/8 signaling in HUVECs.

Methods

As described in section 3.2.

Results

4.3.1 Activation of cAMP Generation

To determine if cAMP plays a role in vasculogenesis or phosphorylation of SMAD1/5/8 we first assessed whether GPER activation leads to cAMP generation in HUVECs. Therefore we used a direct competitive cAMP ELISA. HUVECs were pre-treated with mm-IBMX (100 nmol/L), a phosphodiesterease inhibitor, before the addition of G15 (100 nmol/L) or G1 (100 nmol/L). Activation of GPER with G1 induced cAMP generation from 100±2% to 122±3.3% ($p<0.05$ relative to control), and this effect was significantly abrogated by G15 to 87±4.5% ($p<0.05$ relative to G1 treated HUVECs, Figure 27). Since GPER activation induced cAMP generation in HUVECs, it may play an important role in vasculogenesis via the phosphorylation of SMAD1/5/8.

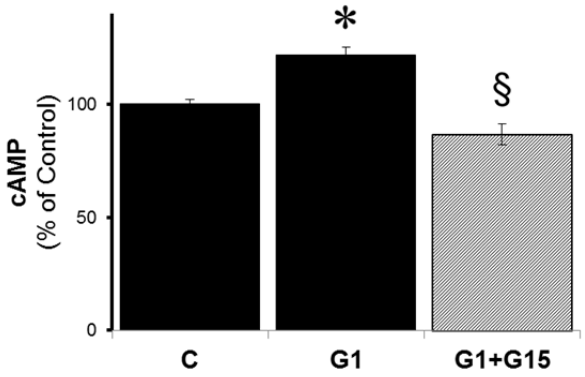

Figure 27. Bar graph of direct cAMP ELISA, demonstrating the effects of GPER specific agonist (G1; 100 nmol/L) and GPER specific antagonist (G15; 100 nmol/L) on the generation of cAMP. HUVECs were starved overnight and pre-treated with PDE-inhibitor mm-IBMX (100 nmol/L) for 10min, followed by G15 for 10 min and G1 for additional 15 min. ELISA was performed according to protocol, including the acetylation step, and measured at the TECAN plate reader at 405 nm. The cAMP concentration (pmol/mg) was normalized to total protein concentration (mg/ ml), obtained by BCA. The corresponding value for the control (100%) is 18.12 pmol cAMP/ mg of total protein. Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to G1, using ANOVA test.

4.3.2 GPER-induced Capillary Formation and SMAD1/5/8 Phosphorylation depends on cAMP/ PKA

To study the impact of cAMP-signaling on the ALK1/ SMAD1/5/8 pathway and vasculogenesis in HUVECs we employed different pharmacological inhibitors, i.e. DDA, an adenylate cyclase specific inhibitor, and PKI (5-24), a PKA-specific inhibitor. As shown in Figure 28A, G1 (10 nmol/L)-induced phosphorylation of SMAD1/5/8 was significantly inhibited in the presence of DDA (1 µmol/L) from 210±17.4% to 65±7.9% ($p<0.05$ relative to G1 treated HUVECs). Similar results were obtained for capillary formation, where DDA (1 µmol/L) abrogated the stimulatory effects of G1 by 83% ($p<0.05$ relative to G1 treated HUVECs, Figure 28B). Next, we applied PKI (5-24) (10 nmol/L) and observed a decrease in G1-induced pSMAD1/5/8 from 205±16.4% to 100±7.9% and capillary formation from 141±5.7% to 78±13.2% ($p<0.05$ relative to G1 treated HUVECs, Figure 28C/ D). These findings provide evidence that Gαs-Adenylylcyclase-cAMP is the signaling cascade via which GPER induces SMAD1/5/8 phosphorylation and capillary formation in HUVECs. However, based on our findings, the precise mechanism by which PKA targets the ALK1/ SMAD1/5/8 pathway remains unclear.

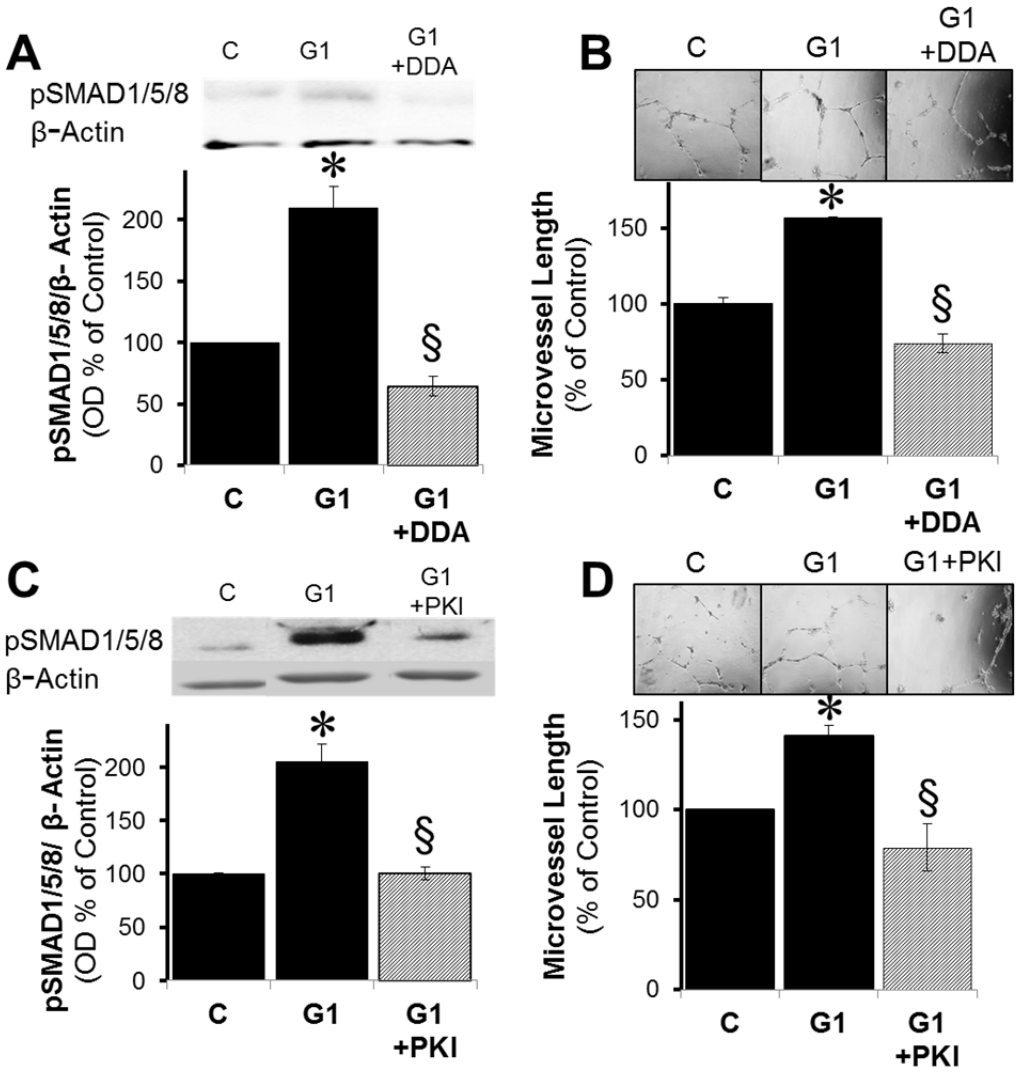

Figure 28. Panel A, Bar graph and representative Western Blot, demonstrating effects of GPER specific agonist (G1; 10 nmol/L) and Adenylate cyclase specific inhibitor (DDA, 1μmol/L) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs were treated with the inhibitor for 30 min, and subsequently G1 was added for additional 45 min. Values represent mean±SEM, n=4, *P<0.05, relative to control, §P<0.05 relative to G1, using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L), and Adenylate cyclase specific inhibitor (DDA, 1μm/L) on capillary formation by HUVECs. Cells were plated in a density of 4000 cells per well on a matrigel–coated 15-well μ- slides and pretreated with the inhibitor for 30 min, subsequently G1 was added. After incubation overnight the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=4, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test. **Panel C,** Bar graph and representative Western Blot, demonstrating effects of GPER specific agonist (G1; 10 nmol/L) and PKA inhibitor PKI (5-24) (PKI, 10 nmol/L) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs were pretreated with inhibitor for 30 min, subsequently G1 was added for additional 45 min. Values represent mean±SEM, n=5, *P<0.05, relative to control, §P<0.05 relative to G1 using ANOVA test. **Panel D,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L) and PKA inhibitor PKI (5-24) (PKI, 10 nmol/L) on capillary formation. Cells were plated in a density of 4000 cells per well on a matrigel–coated 15-well μ- slides and pretreated with inhibitor for 30 min, and subsequently BMP2 was added. After incubation overnight the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=4, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

4.3.3 Role of cAMP/ PKA in Capillary Formation and ALK1/ SMAD1/5/8 Pathway

To investigate whether ALK1 or pSMAD1/5/8 is a target of PKA in HUVECs, we used DBcAMP (a stable autolog of the endogenous cAMP), Noggin (the endogenous antagonistic ligand of BMP receptor type I) and ALK1Fc (the specific antagonizing antibody for ALK1). We first studied the role on pSMAD1/5/8 and observed induction upon DBcAMP (100 µg/ml) treatment to 221±21.13% ($p<0.05$ relative to control). ALK1Fc (100 ng/ml) significantly abrogated DBcAMP-induced phosphorylation of SMAD1/5/8 from 221±21.13% to 98±37.6%, whereas Noggin (100 ng/ml) further increased the stimulatory effects of DBcAMP to 287±56.1 % ($p<0.05$ relative to DBcAMP treated HUVECs, Figure 29A). Next, the modulatory effects of the same antagonists were tested on DBcAMP-induced microvessel formation by HUVECs. DBcAMP (100 µg/ml) treatment induced capillary formation by 74%±10.9% ($p<0.05$ relative to control), and this effect was abrogated by ALK1Fc (100 ng/ml) to 84±7.4% but not altered by Noggin (100 ng/ml) to 173±8.5% ($p<0.05$ relative to DBcAMP treated HUVECs, Figure 29B). Based on these findings the participation of TGFβ receptor II, as a target of PKA, can be excluded, because the effects of DBcAMP on SMAD1/5/8 phosphorylation or capillary formation were not altered by Noggin. In contrast, ALK1Fc decreased both DBcAMP-induced pSMAD1/5/8 and capillary formation, suggesting that PKA directly activates ALK1 and therefore stimulates SMAD1/5/8 signaling and vasculogenesis in HUVECs.

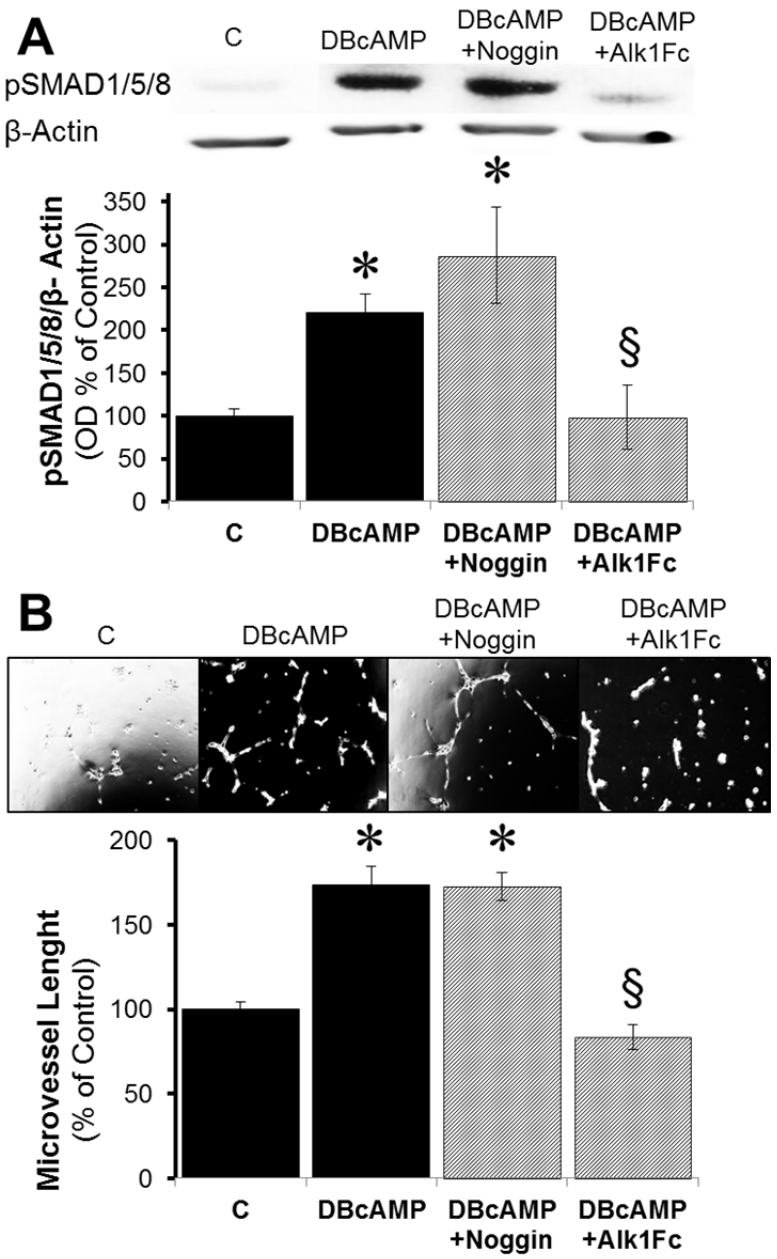

Figure 29. Panel A, Bar graph and representative Western Blot, demonstrating the effects of cell permeable cAMP analogue (DBcAMP; 100µg/ml), BMP inhibitor (Noggin, 100 ng/ml) and ALK1 specific antagonizing Antibody (Alk1Fc; 100 ng/ml) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs were treated with the inhibitors for 30 min, subsequently DBcAMP was added for additional 45 min. Values represent mean±SEM, n=3, *P<0.05, relative to control, §P<0.05 relative to DBcAMP, using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of cell permeable cAMP analog (DBcAMP; 100 µg/ml), BMP inhibitor (Noggin, 100 ng/ml) and ALK1 specific antagonizing Antibody (Alk1Fc; 100 ng/ml) on capillary formation by HUVECs. Cells were plated at a density of 4000 cells per well on a matrigel–coated 15-well µ-slides and pretreated with the inhibitors for 30min, subsequently DBcAMP was added. After incubation overnight capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to DBcAMP using ANOVA test.

Discussion

The second messenger cAMP is an omnipresent signal transducer for cells and its production is induced upon estrogen treatment [104, 233-236]. Upon binding of a ligand to a GPCR coupled to a Gαs subunit, cAMP is synthesized from adenosine triphosphate (ATP) by the enzyme adenylyl cyclase. Generation of cAMP in turn activates several proteins like nucleotide-gated ion channels and protein kinase A (PKA). Subsequently, PKA phosphorylates effector proteins at serine or threonine residues and therefore activates them [232]. These activated effector proteins further mediate the inhibitory actions on the proliferation of SMCs [104, 291], vasodilation of arteries *in vivo* [236, 290, 295] and the improvement of endothelial function by inducing barrier function [296, 297]. Additionally, membrane-permeable cAMP analogues have been shown to induce VEGF expression and activate the PI3K/Akt and eNOS/NO pathway, thereby inducing angiogenesis [191, 237-239]. Based on these findings, it is generally accepted that the protective effects of estrogens on the cardiovascular system are in part mediated by the induction of cAMP signaling [104, 236, 289-291]. Recent studies provide evidence that estrogen signaling via GPER induces cAMP production in MCF7 cells [39, 41], murine pancreatic islets [294] and VSMCs of hypertensive mREN2.Lewis rats [295].

To our knowledge, to date, there are no reports demonstrating that GPER stimulates cAMP signaling in ECs. Hence we investigated whether GPER activation results in cAMP production by cultured HUVECs. We quantified cAMP levels following ligand treatment by using a direct competitive ELISA and observed a significant increase in cAMP in HUVECs treated with G1. This G1-induced cAMP formation was inhibited by pre-treatment with GPER specific antagonist G15, suggesting a specific role of GPER in the induction of cAMP in HUVECs. Consistent with the findings of other studies [39, 41, 294, 295], our results indicate that GPER activation increases cAMP levels. More importantly, this is the first study to report induction of cAMP production in endothelial cells following GPER activation.

Several studies have shown that cell-permeable analogues of cAMP induce the expression of VEGF and activate pro-angiogenic signaling such as the PI3K/ Akt and the eNOS/ NO pathway, thereby inducing angiogenesis [191, 237-239]. Hence, we decided to investigate whether cAMP mediates GPER induced ALK1/ SMAD1/5/8 signaling and vasculogenesis in HUVECs. To achieve this goal, pharmacological

inhibitors for the components of the cAMP signaling pathway, i.e. for adenylyl cyclase and for PKA, were applied. We were able to block G1-induced SMAD1/5/8 phosphorylation and G1- stimulated capillary formation with DDA, a specific adenylyl cyclase inhibitor, and with PKI (5-24), a specific PKA inhibitor. These findings indicate that GPER-induced vasculogenesis and phosphorylation of SMAD1/5/8 in HUVECs are potentially mediated via cAMP signaling.

In this context, De Lorenzo et al. reported decreased tumorigenesis in adenylyl cyclase deficient mice [298] and Namkoong et al. reported that forskolin-induced angiogenesis was impaired after PKA-inhibitor PKI application [239]. In line with these findings and several other studies showing that cAMP and estrogens are important for capillary formation in ECs [100, 191, 237-239, 250], we postulate that cAMP is involved in GPER-induced vasculogenesis in HUVECs.

Furthermore, our observation that DBcAMP mediates G1-stimulated phosphorylation of SMAD1/5/8 is supported by the finding that cAMP enhances BMP4-induced gene expression of ID-1, which is the target gene of pSMAD1/5/8 [299]. Moreover, Ghayor et al. reported that cAMP increases BMP2 signaling in osteoclasts via PKA and MKP1 dependent mechanism [300]. The fact that the promoter region of the *ID-1* gene contains both, a BMP-responsive element BRE and a cAMP responsive element CRE [301-303], suggests a synergy between cAMP and TGFβ/ BMP signaling and, as well as the other above findings, support our hypothesis that cAMP might partly be responsible for GPER induced ALK1/ SMAD1/5/8 signaling. More experiments, including the assessment whether cAMP induces BMP2 secretion, are needed to fully elucidate this synergy/ crosstalk between cAMP and TGFβ/ BMP/ ALK1/ SMAD1/5/8 in ECs.

ALK1 is activated following phosphorylation of serine and threonine residues at the GS domain by TGFβRII. Upon activation, ALK1 recruits and phosphorylates SMAD1/5/8 at the C-terminal serine 463 and serine 465 residues [304], leading to the formation of complex with SMAD4. This complex translocates into the nucleus and activates transcription of the target gene *ID-1* [145-147], which induces migration, proliferation and tube formation in ECs [149, 153]. Activation of PKA by cAMP results in phosphorylation of effector proteins at serine or threonine residues and their activation [232]. Hence, it is possible that PKA also catalyzes the phosphorylation of ALK1 and/ or SMAD1/5/8. With regard to the observation that pharmacological

inhibition of PKA reduced G1-stimulated pSMAD1/5/8, we hypothesize that PKA might play a role in activating ALK1 or SMAD1/5/8.

To distinguish whether PKA targets ALK1 or SMAD1/5/8, we applied DBcAMP (analogue of endogenous cAMP), Noggin (endogenous antagonistic ligand of BMP receptor type I) and ALK1Fc (specific antagonizing antibody for ALK1). Capillary formation and phosphorylation of SMAD1/5/8 were significantly stimulated with DBcAMP treatment in HUVECs. Moreover, pre-treatment with ALK1Fc significantly decreased DB-cAMP induced vasculogenesis and SMAD1/5/8 phosphorylation, whereas Noggin had no effect. These results suggest that cAMP-activated PKA stimulates ALK1, but not SMAD1/5/8. However, it is possible that cAMP induces BMP production/ secretion and thereby activates ALK1/ SMAD1/5/8 signaling. But the fact, that Noggin blocked the stimulatory effects of DBcAMP on capillary formation and SMAD1/5/8 phosphorylation, argues against a role of BMP in mediating these effects. Still, further experiments are required to rule out a potential production/ secretion of BMP upon treatment with DB-cAMP in HUVECs. Ji and Andrisani et al. showed that PKA stimulates pSMAD1 by activating ERK, which in turn leads to phosphorylation of SMAD1 [305], supporting our notion that cAMP- stimulated PKA may activate ALK1/ SMAD1/5/8 signaling.

In summary, the above findings indicate that there is a functional crosstalk between cAMP/ PKA and TGFβ signaling. However, more in depth research is needed to fully elucidate the interactions within this signaling cascade. Our results, obtained in HUVECs, suggest that PKA activates ALK1 directly, leading to SMAD1/5/8 phosphorylation and thereby stimulating vasculogenesis. More importantly, we propose that GPER induced cAMP production activates PKA and thus initiates the ALK1/ SMAD1/5/8 signaling cascade, leading to increased capillary formation in HUVECs.

4.4 The PI3K/ Akt Pathway mediates GPER-induced Capillary Formation in Endothelial Cells

Objective

The PI3K/ Akt pathway is a prominent pathway, which regulates multiple cellular functions including metabolism, growth, proliferation, survival, transcription, protein synthesis and angiogenesis. Since GPER activation induced capillary formation, we wanted to investigate whether GPER activates PI3K/ Akt signaling pathway is involved in GPER-induced vasculogenesis and ALK1/ SMAD1/5/8 signaling.

Introduction

The PI3K/ Akt pathway plays a central role in controlling endothelial cell viability [180, 181]. Migration and formation of capillary like structures is essential for endothelial cells to form angiogenesis *in vivo.* Moreover, Akt protein accumulates in endothelial tip cells with increased migratory abilities [193]. The important role of the PI3K/ Akt pathway in mediating angiogenesis has been supported by several studies [181, 189, 190]. Due to its mitogenic and angiogenic role, PI3K/ Akt signaling is also involved in tumorigenesis and regulates estrogen induced cell proliferation in ER+ breast cancer [194, 195] and endometrial cancer [196]. Importantly, specific PI3K/ Akt inhibitors are used clinically as anti-angiogenic agents in cancer [197, 198] and breast cancer therapy [195]. Furthermore, estrogen stimulated angiogenesis has been reported to be mediated via the PI3K/ Akt pathway [100, 240, 241]. Recently, it has also been shown that GPER mediated estrogen signaling promotes survival of MCF-7 cells [306] and migration of renal carcinoma cells in a PI3K/ Akt dependent fashion [307]. Taken together these findings implicate PI3K/ Akt as a key player in angiogenesis.

Hence, in the present study we assessed whether GPER activation induces PI3K/ Akt signaling in HUVECs and stimulates vasculogenesis. Moreover, we studied whether there is an association between the two pro-angiogenic pathways PI3K/ Akt and ALK1/ SMAD1/5/8 in our HUVECs model.

Methods

As described in Section 3.2.

Results

4.4.1 Activation of PI3K/ Akt Pathway

Treatment with G1 (10 nmol/L) significantly induced Akt phosphorylation in HUVECs from 100% to 178±24.4% ($p<0.05$ relative to control). Upon pre-treatment with G15 (100 nmol/L), G1-induced Akt phosphorylation was reduced to 43±8.6% ($p<0.05$ relative to G1 treated HUVECs, Figure 30A). The regulatory role of GPER on Akt phosphorylation was further confirmed in GPER-silenced HUVECs, in which the treatment with G1 (10 nmol/L) and E2 (10 nmol/L) mildly attenuated the phosphorylation of Akt from 223±72.9% to 169±30.1% and 127±12.5%, respectively ($p>0.05$ relative to GPER silenced control). Whereas, G1 and E2 induced the phosphorylation of Akt in HUVECs, treated with scrambled siRNA, from 100% to 162±31.2% and 127±12.5%, respectively ($p<0.05$ relative to scrambled control, Figure 30B).

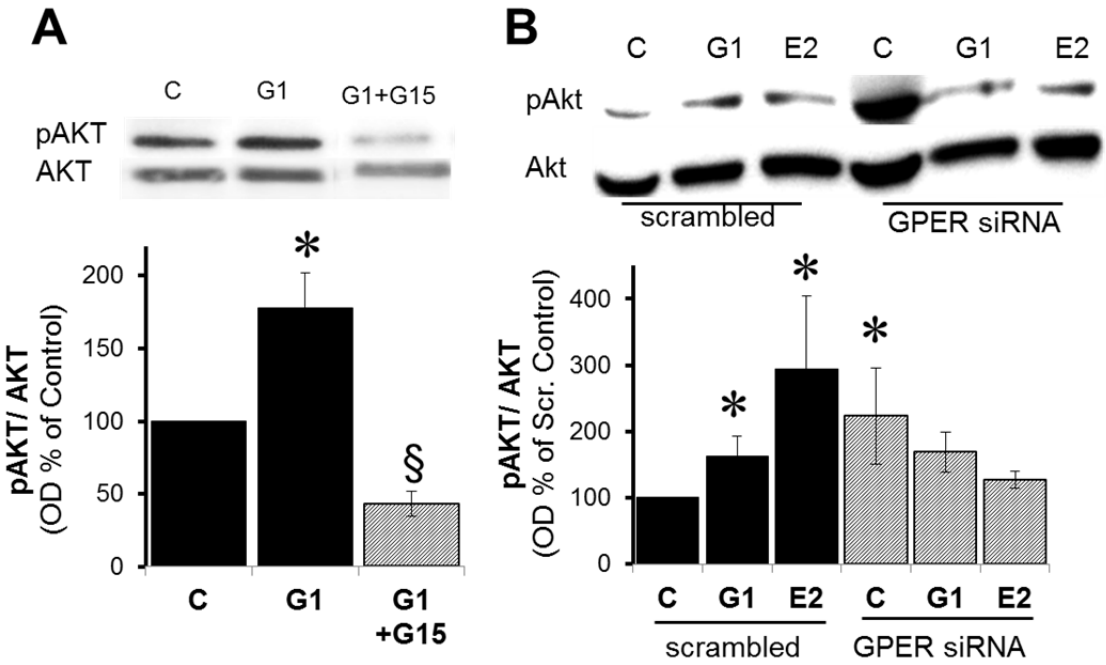

Figure 30. Bar graphs and representative Western Blots showing the effect on Akt phosphorylation **Panel A,** Effects of GPER agonist (G1; 10 nmol/L) and GPER antagonist (G15; 100 nmol/L), **Panel B,** Effects of GPER specific agonist (G1; 10 nmol/L) and estradiol (E2; 10 nmol/L) in HUVECs, transfected with scrambled siRNA (50 nmol/L) or GPER siRNA (50 nmol/L). Pre-starved HUVECs were treated for 30 min with the inhibitor, followed by additional 45 min with G1. Values represent mean±SEM, n=3, *$P<0.05$ relative to control or scrambled control, §$P<0.05$ relative to G1 treated HUVECs using ANOVA test.

4.4.2 Role of PI3K/ Akt Pathway in GPER-induced Capillary Formation

Our findings that GPER activation induces the PI3K/ Akt pathway, together with the fact that PI3K/ Akt induces capillary formation by ECs led us to postulate and assess the role of G1- induced Akt signaling in mediating vasculogenesis of HUVECs.

In order to scrutinize this role, we utilized the pharmacological PI3K inhibitor LY294002 (LY; 5 µmol/L). First we assessed the impact on G1 (10 nmol/L) -induced Akt phosphorylation, which was abrogated upon LY treatment from 194±20% to 77±20.8% ($p<0.05$ relative to G1 treated HUVECs, Figure 31A). Similar results were obtained in capillary formation, where the stimulatory effect of G1 was reduced by LY from 152±3.7% to 54±18.5% ($p<0.05$ relative to G1 treated HUVECs, Figure 31B). Our data suggests that GPER-induced vasculogenesis is mediated via the activation of the prominent pro-angiogenic PI3K/ Akt pathway.

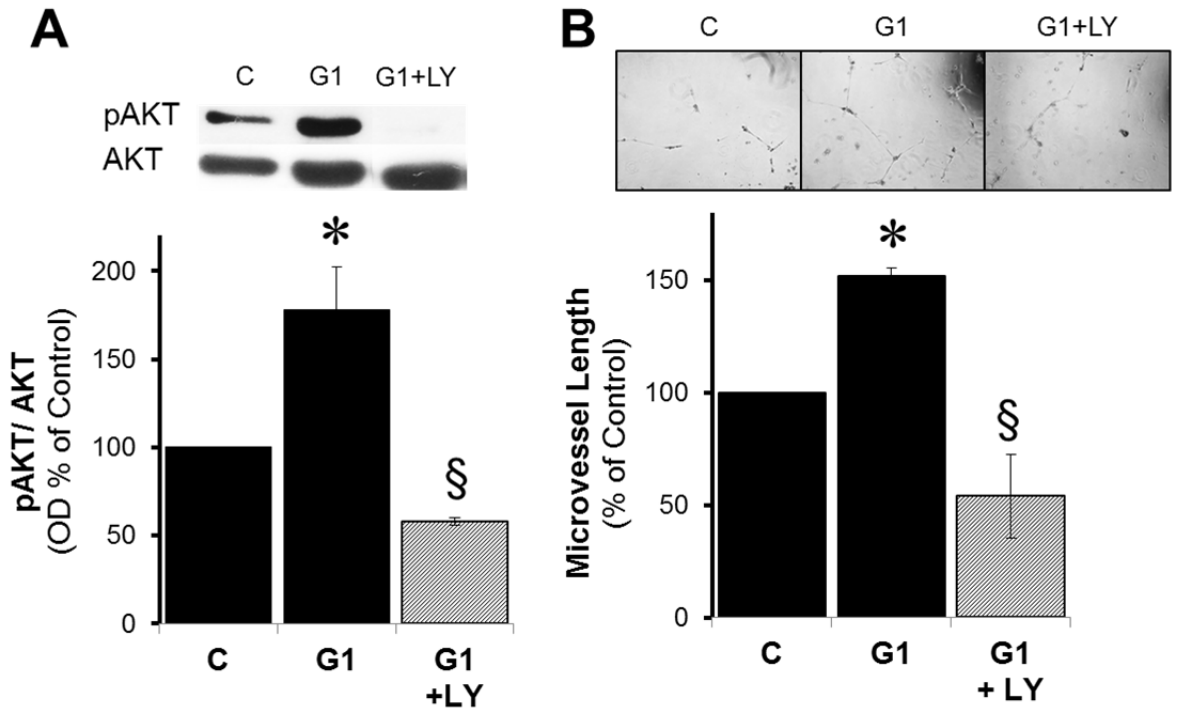

Figure 31. Bar graphs, representative Western Blot and representative photomicrographs showing the effects on the phosphorylation of Akt and capillary formation in HUVECs. **Panel A,** Effect of GPER agonist (G1; 10 nmol/L) +/- PI3K-inhibitor LY294002 (LY; 5 µmol/L) on the phosphorylation of Akt. Pre-starved HUVECs were treated for 30 min with the inhibitor, followed by 45 min with G1. Values represent mean±SEM, n=3, *P<0.05 relative to control §P<0.05 relative to G1 using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L) and PI3K-inhibitor (LY294002; 5 µmol/L) on capillary formation. The cells were plated at a density of 4000 cells per well on a matrigel–coated 15-well µ- slides and pretreated with the inhibitor for 30min, G1 was then added. After incubation overnight, capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=5, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

4.4.3 GPER-induced Capillary Formation depends on Crosstalk between ALK1/ SMAD1/5/8 and PI3K/ Akt Pathway

Our findings show that GPER- induced vasculogenesis is mediated via two important pro-angiogenic pathways: the ALK1/SMAD1/5/8 and the PI3K/Akt pathway. Vasculogenesis is a complex, fragile action of ECs and involves many different signaling pathways and cellular mechanisms. Since both pathways are important for GPER-induced vasculogenesis, we examined whether there is a link between ALK1/ SMAD1/5/8 and PI3K/ Akt pathways in HUVECs.

To accomplish this goal we utilized ALK1Fc (100 ng/ml) a specific antagonizing antibody and pharmacological inhibitor for ALK1, and SJN2511 (100 nmol/L), a specific antagonist for ALK5, and assessed their impact on phosphorylation of Akt in HUVECs. As shown in Figure 32A, treatment with G1 (10 nmol/L) induced Akt phosphorylation and this stimulatory effect was significantly abrogated by Alk1Fc from 178% to 79±13.1% ($p<0.05$ relative to G1 treated HUVECs), whereas SJN did not alter G1-induced Akt phosphorylation, which was 178% and 174±25% in absence and presence of SJN ($p>0.05$ relative to G1 treated HUVECs). To further investigate the possible link, we silenced SMAD1 in HUVECs, by transfecting them with SMAD1 specific siRNA (50 nmol/L). Upon treatment with BMP9 (10 ng/ml) phosphorylation of Akt was significantly reduced in SMAD1-silenced HUVECs as compared to HUVECs transfected with scrambled siRNA, from 100% in scrambled control to 50±18.7% in SMAD1 silenced ($p<0.05$ relative to scrambled control, Figure 32B). Next, we applied the pharmacological PI3K inhibitor LY294002 (LY; 5 µmol/L) prior to G1-treatment and observed a significant decrease in phosphorylation of SMAD1/5/8 by 117% ($p<0.05$ relative to G1 treated HUVECs, Figure 32C). Taken together, these observations suggest a crosstalk between ALK1/ SMAD1/5/8 and PI3K/ Akt, moreover we hypothesize that this interaction is at the level of SMAD1/5/8 and Akt, as silencing of SMAD1 abrogated phosphorylation of Akt.

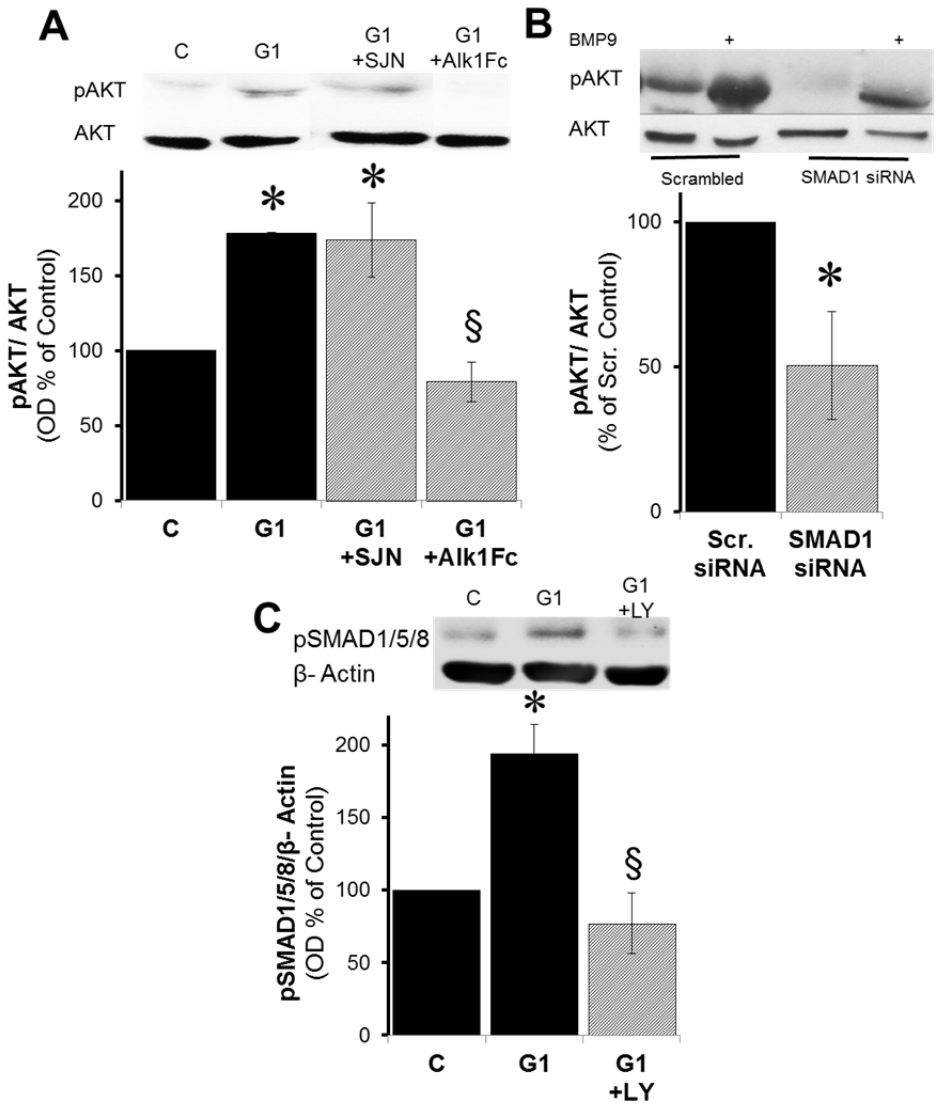

Figure 32. Bar graphs and representative Western Blots demonstrating the effects on phosphorylation of AKT in HUVECs by (**Panel A),** GPER specific agonist (G1; 10 nmol/l)+/- ALK 5 specific antagonist (SJN; 100 nmol/L) or ALK 1 specific antagonizing antibody (Alk1Fc; 100 ng/ml); and effects of (**Panel B),** BMP9 (10 ng/ml) in cells**,** transfected with SMAD1 specific siRNA (50 nmol/L) or scrambled siRNA (50 nmol/L). HUVECs were starved and pre-treated with the antagonists for 30 min, G1 or BMP9 was then added for 45 min. **Panel C,** Bar graph and representative Western Blot showing the effects of GPER agonist (G1; 10nmol/L) and PI3K inhibitor LY294002 (LY; 5 µmol/L) on the phosphorylation of SMAD1/5/8. Pre-starved HUVECs treated for 30 min with the inhibitor, followed by 45min with G1. Values represent mean±SEM, n=3, *P<0.05 relative to control or scrambled control, §P<0.05 relative to G1, using ANOVA test.

4.4.4 GPER-induced Capillary Formation and SMAD1/5/8 Phosphorylation does not depend on MMP

One of the signaling cascades leading to PI3K/Akt activation is the Matrix Metallo Proteinase (MMP). Stimulation of MMP induces EGF release and activates the EGF-Receptor, which subsequently stimulating MAPK or PI3K and their downstream signals. To completely understand the role of PI3K/ Akt in GPER-induced

vasculogenesis and phosphorylation of SMAD1/5/8 we investigated the impact of MMP. To accomplish this, we applied Batimastat (B; 5 µm/L), the MMP specific inhibitor. As shown in Figure 33A/ B, treatment with B mildly increased G1 (10 nmol/L)-induced phosphorylation of SMAD1/5/8 and capillary formation from 255±35.2% to 344±34% and from 157±0.5% to 166±18%, respectively ($p<0.05$ relative to control). These results reveal that GPER-induced vasculogenesis and phosphorylation of SMAD1/5/8 is not dependent of MMP, even though it is known to activate PI3K/ Akt signaling.

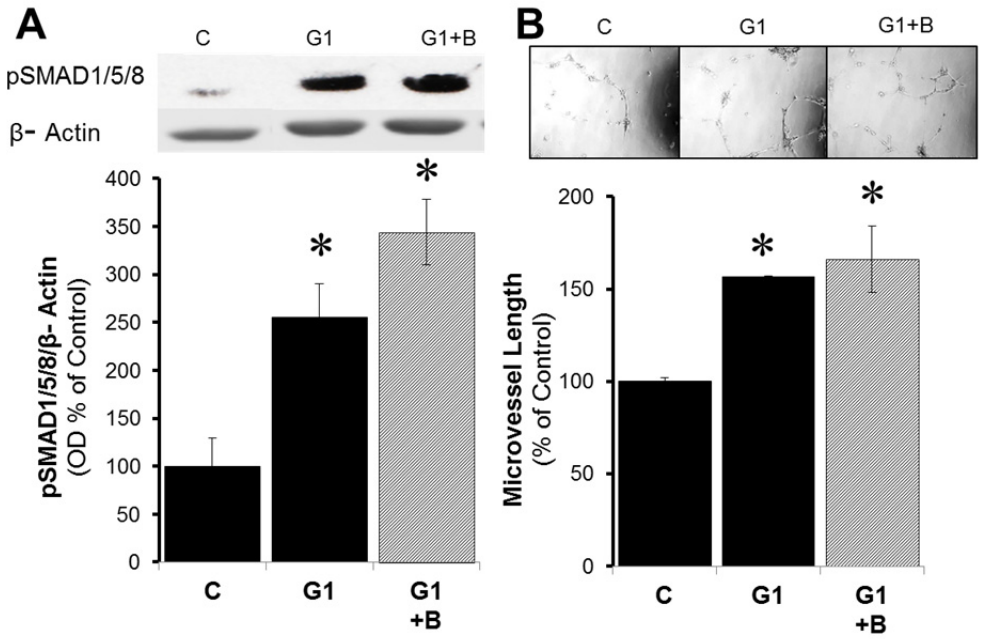

Figure 33. Panel A, Bar graph and representative Western Blot demonstrating the effects of GPER specific agonist (G1; 10 nmol/l) and MMP specific inhibitor Batimastat (B; 5 µm/L) on SMAD1/5/8 phosphorylation. HUVECs were starved and pre-treated with the antagonists for 30 min and G1 was then added for 45 min. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test. **Panel B,** Bar graph and representative photomicrographs showing the effects of GPER specific agonist (G1; 10 nmol/L) and MMP specific inhibitor Batimastat (B; 5 µm/L) on capillary formation by HUVECs. The cells were plated at a density of 4000 cells per well on a matrigel–coated 15-well µ-slides and pretreated with the inhibitor for 30min, G1 was then added. After incubation overnight the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test.

Discussion

The PI3K/ Akt/ pathway is highly conserved and controls multiple cellular functions including cell metabolism, growth, proliferation, survival, transcription, protein synthesis and angiogenesis [180, 181]. Several studies have reported the important regulatory role of PI3K/ Akt in postnatal blood vessel formation and processes related to angiogenesis [181, 189, 190]. Furthermore, the PI3K/ Akt pathway has been

shown to be an important mediator of estrogen stimulated angiogenesis in ECs [100, 240, 241]. Very recently, it was reported that GPER mediated estrogen signaling is transduced via the PI3K/ Akt pathway and induces survival of MCF-7 cells [306] and migration of renal carcinoma cells [307].

Therefore, the aim of this study was to investigate whether GPER activation induced PI3K/ Akt signaling in HUVECs. Our finding that treatment with GPER specific agonist G1 significantly increased Akt phosphorylation, suggests that G1 activates PI3K/ Akt signaling. This is in line with the finding of Guan et al., who showed that G1 treatment specifically increases Akt phosphorylation in renal carcinoma cells, thereby promoting metastasis [307]. Our observation that GPER specific antagonist G15 significantly inhibited G1-induced Akt phosphorylation, indicates a prominent and specific role of GPER in activating PI3K/ Akt signaling. This is further supported by the finding that in HUVECs, transfected with GPER specific siRNA, the stimulatory effects of G1 and E2 were lost. In contrast to GPER siRNA treated HUVECs, G1 and E2 significantly induced Akt phosphorylation in HUVECs, transfected with scrambled siRNA. Interestingly, compared to HUVECs treated with scrambled controls, pAKT was significantly upregulated in GPER silenced controls. Other studies using GPER siRNA have observed a decrease in G1-stimulated proliferation of CAFs [258] and reduction in proliferation, migration and invasion of SKOV3 cells [259]. These contrasting outcomes could possibly be due to the use of different cell lines. Moreover, contribution of the use of different GPER siRNAs as a possible explanation can be excluded as pooled GPER siRNAs were used in the present study.

GPER is coupled to heterotrimeric G-proteins Gα/ Gβ/ Gγ, each exhibiting specific signaling cascades. Different subtypes exist from these heterotrimeric G-proteins and for GPER the subtypes Gαs [41] and Gβγ [38] have been proposed. With regard to our results, we suggest that upon silencing of GPER, still unknown pro-angiogenic or pro-survivor subunits of GPER may have been exposed, thereby leading to the stimulation of Akt phosphorylation in HUVECs. Nevertheless, silencing of GPER abrogated the stimulatory effects of G1 and E2, indicating the importance of GPER in mediating the stimulation of Akt phosphorylation by estrogens in HUVECs.

Although the regulatory role of PI3K/ Akt signaling in estrogen-mediated angiogenesis has already been proven [100, 240, 241], it is still not known whether

this pathway mediates GPER-stimulated capillary formation. Hence, we investigated whether PI3K/ Akt signaling regulates G1-induced vasculogenesis in HUVECs. Using a specific PI3K inhibitor, LY294002, we observed a significant inhibition of both, G1-induced phosphorylation of Akt and G1-stimulated vasculogenesis in HUVECs. These observations are consistent with other reports, demonstrating the inhibitory effect of LY294002 on G1-induced upregulation of MMP9 in AHCN and OS-RC-2 cells [307]. Furthermore, application of wortmannin, another specific PI3K inhibitor, significantly abolished G1-mediated improvement of functional recovery and reduced infarct size in isolated hearts of Sprague Dawley rats [109]. In summary, these findings indicate that the PI3K/ Akt pathway plays a potential role in GPER-mediated estrogen signaling. The PI3K/ Akt pathway is known to be an important pro-angiogenic pathway and PI3K inhibition may generally inhibit vasculogenesis, also in response to other factors. But the fact that G1- induced phosphorylation of Akt was inhibited by pre-treatment with G15, suggests that GPER-stimulated vasculogenesis in HUVECs is, in part, mediated by PI3K/ Akt signaling.

Recently, TGFβ pathway components have been shown to interact with other signaling pathways [308]. In section 4.2 we showed that GPER-activation induces ALK1/ SMAD1/5/8 signaling and in this section we demonstrate that GPER-activation promotes PI3K/ Akt signaling, suggesting that both pathways are partly responsible for G1-stimulated vasculogenesis. Hence, we further assessed whether GPER-mediated PI3K/ Akt and ALK1/ SMAD1/5/8 interact with/ or regulate each other in HUVECs. To investigate this interaction/ regulation, we employed ALK1Fc (neutralizing antibody for ALK1), SJN2511 (specific ALK5- inhibitor) and LY294002 (specific PI3K inhibitor). Our findings that G1-induced Akt phosphorylation was significantly inhibited by ALK1Fc, whereas SJN had no modulatory effects on the phosphorylation of Akt, provides evidence for an interaction between GPER-induced PI3K/ Akt signaling with ALK1/ SMAD1/5/8 but not ALK5 signaling. This contention is further supported by the fact that silencing of SMAD1 in HUVECs was accompanied with a significant reduction in pAkt upon BMP9 treatment; moreover, G1-induced phosphorylation of SMAD1/5/8 was abolished upon pre-treatment with LY294002. Taken together, our results suggest that both GPER-induced pro-vasculogenic pathways PI3K/ Akt and ALK1/ SMAD1/5/8 interact with each other to induce vasculogenesis by HUVECs. Based on our observations that pAkt is reduced upon SMAD1 silencing, we suggest that the interaction and regulation occurs at the level

of Akt and SMAD1/5/8. Several studies have shown that TGFβ signaling also activates non-SMAD pathways. For example PI3K/ Akt activation [161, 162] has been implicated in TGFβ induced cell survival and epithelial-to-mesenchymal-transition (EMT), which leads to the acquirement of motile and invasive properties [309, 310], needed for angiogenesis. Lee et al. [311] reported activation of PI3K/ Akt signaling upon BMP9 treatment was independent of ALK1 and ALK5, but dependent on endoglin, an accessory type III receptors of TGFβ signaling. In summary, based on our observations and the above findings a crosstalk/ interaction between these two pathways is evident, however more research is needed to dissect the mechanisms. Nevertheless, to our knowledge, our findings provide the first evidence for an interaction between PI3K/ Akt and ALK1/ SMAD1/5/8 and a possible regulation of each other at the level of Akt and SMAD1/5/8 in HUVECs.

Stimulation of MMP by several growth factors triggers EGF release, which activates the EGF receptor and subsequently MAPK or PI3K and their downstream signals [181]. To complete the understanding of PI3K/ Akt signaling in GPER-induced vasculogenesis and GPER-mediated phosphorylation of SMAD1/5/8, we also investigated the impact of MMP. To achieve this goal, we applied Batimastat, a pharmacologically specific inhibitor for MMP, and observed no reduction of G1-induced SMAD1/5/8 phosphorylation and G1-stimulated vasculogenesis. This finding is in contrast to other studies, which showed that Batimastat inhibited *in vivo* angiogenesis in liver metastases of B16F1 melanoma cells [312] and in murine hemangioma [313]. Furthermore, Wang et al. indicated that MMP activates TGFβ signaling in VSMCs of rats [314]. These discordant findings may be due to the use of different experimental models, or due to the effects which are specifically associated with G1 or its impact on MMP. Our findings suggest that MMP may not be involved in GPER-mediated stimulation of SMAD1/5/8 and GPER-induced vasculogenesis, even though the downstream PI3K/ Akt signaling cascade importantly regulates these processes. However, the lack of a positive control for Batimastat activity does not enable us to conclude this for certain. Therefore, the activity of Batimastat has to be assessed using enzymatic activity assays in future studies.

4.5 The Role of NO/ VEGF-A Pathway in GPER-induced Capillary Formation by Endothelial Cells

Objective

Our earlier findings suggest that GPER-induced vasculogenesis is in part dependent on the activation of PI3K/ Akt pathway. PI3K/ Akt signaling is important for generating NO and inducing VEGF-A expression. Both VEGF-A and NO are important endothelial survival factors, which induce EC proliferation, migration and angiogenesis and stimulate/ regulate each other in a reciprocal fashion. Hence, we investigated whether GPER activation induces eNOS activity and NO production and whether GPER upregulates VEGF expression. Moreover, we assessed the role of NO and VEGF on GPER-stimulated vasculogenesis in HUVECs.

Introduction

The formation of new capillaries (angiogenesis or neovascularization) is a carefully balanced process, controlled by a multitude of growth factors and signalling pathways. The fundamental regulatory role of VEGF [191] and NO [192] in angiogenesis has been intensively studied and established. The loss of a single allele of the *VEGF* gene is associated with defective vascularization and embryonic lethality [205, 206], and angiogenic properties of NO are implicated in promoting survival [213, 214], proliferation [215, 216] and migration [217, 218] of endothelial cells. Moreover, the reciprocal interaction of VEGF and NO has been reported to be essential for angiogenesis. NO is both, a downstream and an upstream mediator of VEGF-mediated capillary formation. VEGF activates PI3K/ Akt, leading to activation of eNOS and NO generation. Under normoxic conditions, NO has been shown to induce VEGF expression [188], thereby enhancing EC cell proliferation [215, 216] and inducing capillary formation. More importantly, estrogen induced angiogenesis has been reported to be dependent on VEGF and NO [99]. Studies also suggest that GPER-mediated vasodilation is reliant on endothelium-derived NO, resulting in relaxation of rat aorta, carotid, mesenteric and porcine coronary arteries [108, 116, 117].

Hence, we assessed whether GPER activation induces eNOS phosphorylation, thereby leading to NO production and whether GPER modulates VEGF-A expression

in HUVECs. Furthermore, we investigated the role of NO and VEGF in GPER-induced vasculogenesis in HUVECs.

Methods

As described in section 3.2.

Results

4.5.1 Activation of eNOS and NO Production

We examined whether GPER activation phosphorylates eNOS and leads to increase in NO production by HUVECs. Treatment with G1 (10 nmol/L) induced eNOS phosphorylation from 100% to 149%, and this effect was abrogated by G15 (100 nmol/L) and ICI 182,780 (100 nmol/L) to 47% and 27%, respectively (Figure 34A). Moreover, we observed a significant increase in NO formation by HUVECs treated with G1 (10 nmol/L) and E2 (10 nmol/L), which increased from 100±2.6% to 189±3% and 175±2.3%, respectively ($p<0.05$ relative to control, Figure 34B). In summary, these findings provide evidence that activation of GPER induces eNOS and NO generation.

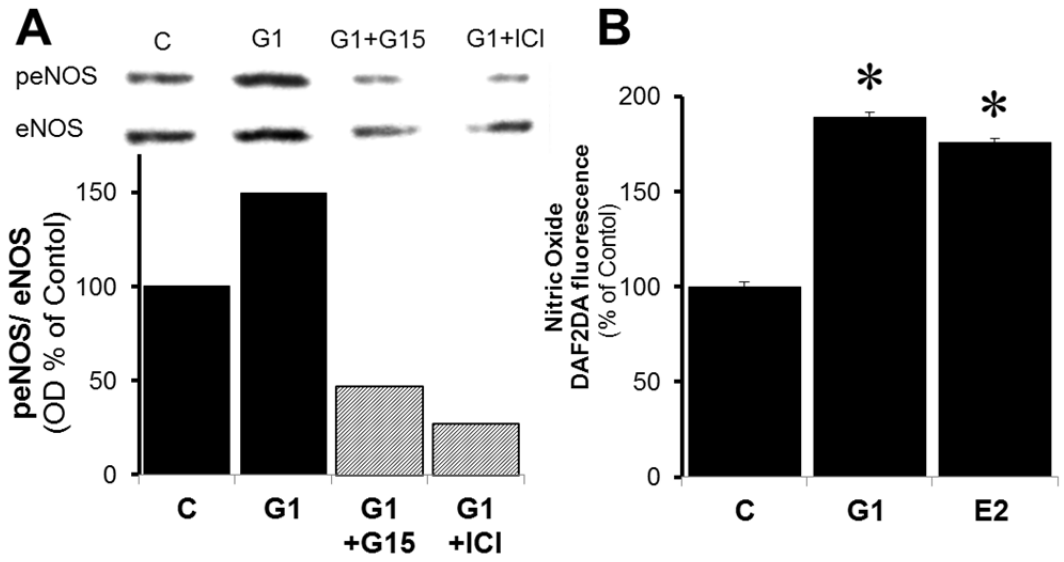

Figure 34. **Panel A,** Bar graph and representative Western Blot showing the effects of GPER specific agonist (G1; 10 nmol/L), in the presence or absence of the GPER specific antagonist (G15, 100 nmol/L) or the ER unspecific inhibitor ICI 182-780 (ICI, 100 nmol/L) on the phosphorylation of eNOS. Pre-starved HUVECs were pretreated with the inhibitors for 30 min, subsequently G1 was added for 45 min. n=1; **Panel B,** Bar graph representing the effects of GPER specific agonist (G1, 10 nmol/L) and estradiol (E2; 10 nmol/L) on NO production. Pre-starved HUVECs were treated with DAF-2DA (1 µmol/L) in the absence of light for 45 min, followed by treatment with G1 or E2 for 15min. Excitation was measured at the TECAN plate reader with 450 nm. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test.

4.5.2 **Activation of VEGF-A Secretion**

Next we studied the impact of GPER activation on VEGF-A protein expression. In HUVECs treated with G1 (10 nmol/L) for 45 min a significant increase in VEGF-A levels was observed and was increased by 97±31.7% ($p<0.05$ relative to control). G1-stimulated VEGF-A expression was inhibited in cells pre-treated with G15 (100 nmol/L) by 118±20% ($p<0.05$ relative to G1 treated HUVECs, Figure 35A). Because the treatment time of 45 min is too short for protein expression, we also assessed the mRNA levels of VEGF-A using RT PCR: As shown in Figure 35B treatment with G1 (10 nmol/L) for 45 min did not modulate VEGF-A mRNA expression, which changed from 1 to 1.08 fold change, while pre-treatment with G15 (100 nmol/L) even increased VEGF-A mRNA to 1.15 fold change ($p<0.05$ relative to control). To exclude translation of VEGF-A mRNA, we pre-incubated the cells with the translation inhibitor Cycloheximide (CY, 10 µmol/L) prior to G1 treatment and examined VEGF-A protein levels. Indeed G1-upregulated VEGF-A protein was not significantly decreased by CY pre-treatment from 145±16.6% to 114±19.7% ($p<0.05$ relative to G1 treated HUVECs, Figure 22C). Our findings suggest that GPER activation does not activate VEGF-A expression, but rather stimulates the release of VEGF-A out of intracellular storage vesicles.

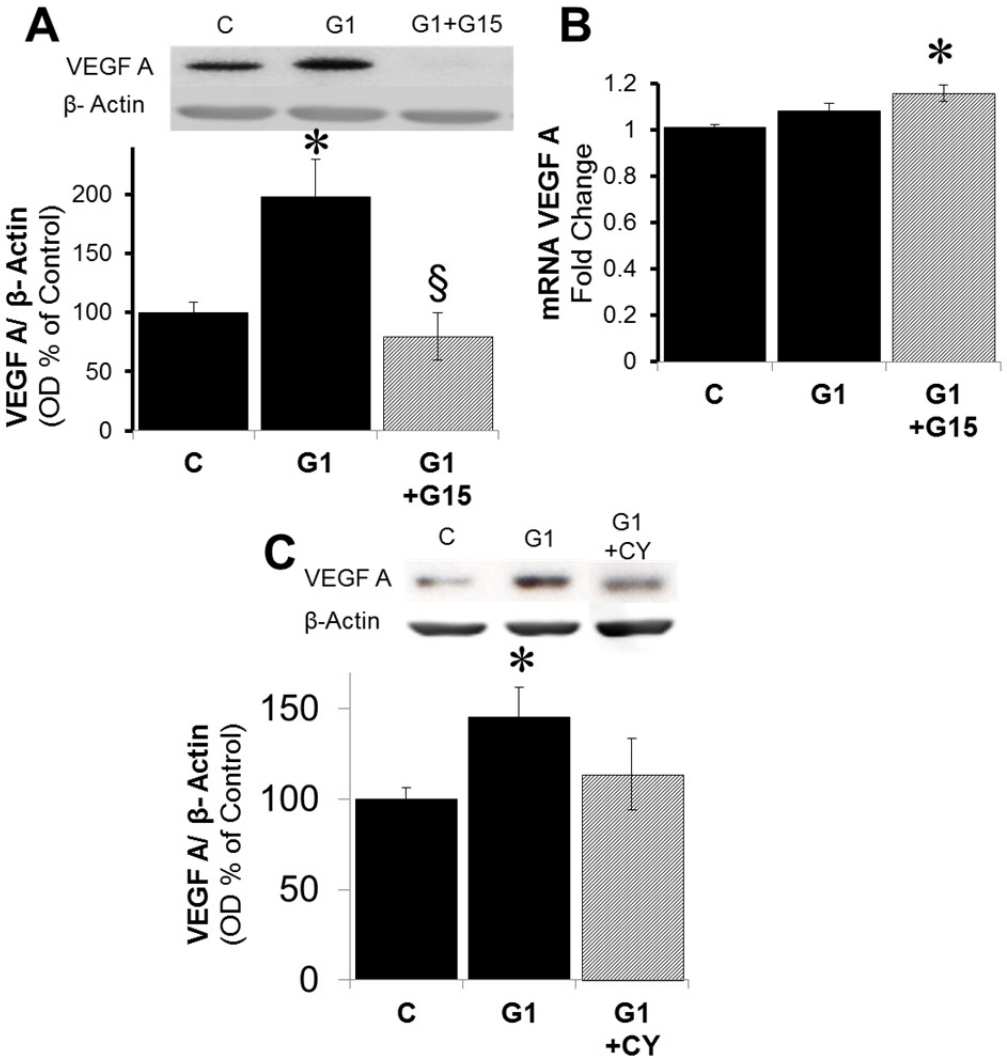

Figure 35. **Panel A,** Bar graph and representative Western Blot showing the effects of GPER specific agonist (G1; 10 nmol/L), in presence and absence of GPER specific antagonist (G15, 100 nmol/L) on VEGF-A- protein expression. Pre-starved HUVECs were treated with G15 for 30 min, and subsequently G1 was added for additional 45 min. Values represent mean±SEM, n=3, *P<0.05, relative to control, §P<0.05 relative to G1 using ANOVA test. **Panel B,** Bar graph representing the effects of GPER specific agonist (G1, 10 nmol/L) +/- GPER specific antagonist (G15, 100 nmol/L) on VEGF-A-mRNA expression. Pre-starved HUVECs were treated with G15 for 30 min, followed by treatment with G1 for 45min. RT-PCR was performed using SYBR green and Primers VEGF-A Fwd: CATGCAGATTATGCGGATCAAAC and VEGF-A REV: GGTCTGCATTCACATTTGTTGTG. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test. **Panel C,** Bar graph and representative Western Blot showing the effects of GPER specific agonist (G1; 10 nmol/L) and translation inhibitor Cycloheximide (CY, 10 µmol/L) on VEGF-A -protein expression. Pre-starved HUVECs were treated with the inhibitor for 30 min, and subsequently G1 was added for another 45 min. Values represent mean±SEM, n=3, *P<0.05, relative to control using ANOVA test.

4.5.3 Role of NO/ VEGF-A Pathway in GPER-induced Capillary Formation

Using different pharmacological inhibitors for eNOS L-NAME (1µmol/L), L-NMMA (1µmol/L) and a specific neutralizing antibody for VEGF-A (500 ng/ml) we assessed the role of NO and VEGF-A in vasculogenesis. As shown in Figure 36 G1 (10 nmol/L)

induced capillary formation, which was significantly inhibited by both NO synthesis inhibitors L-NAME and L-NMMA, from 166±17% to 78±11% and 83±19.3%, respectively ($p<0.05$ relative to G1 treated HUVECs). Similary, treatment with VEGF Ab abrogated G1-induced vasculogenesis from 166±17% to 32±6.3% ($p<0.05$ relative to G1 treated HUVECs). These observations provide evidence that the NO/ VEGF-A pathway is another molecular mechanism, via which GPER activation induces capillary formation by HUVECs. However, additional experiments are required to further confirm whether G1 triggered secretion of VEGF-A is responsible for the long term effects of GPER activated capillary formation by ECs.

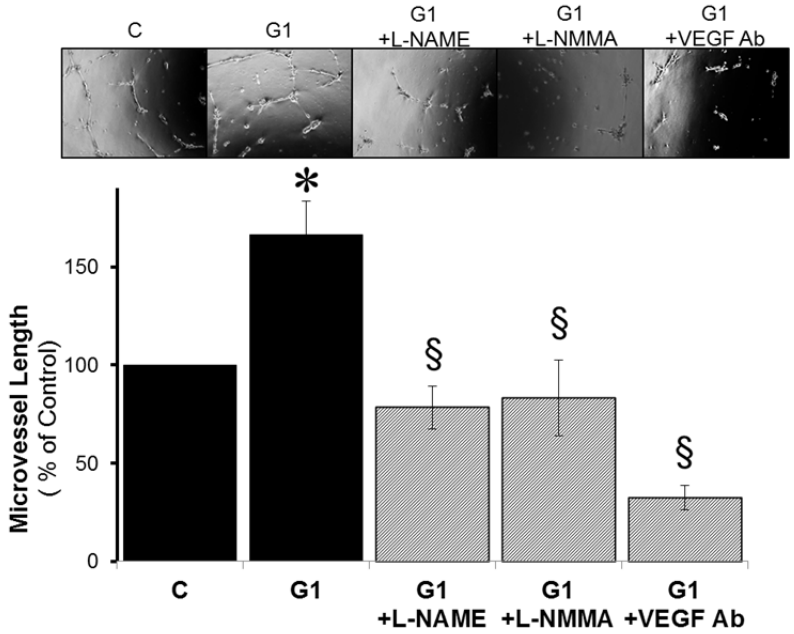

Figure 36. Bar graph and representative photomicrographs showing the effects of **the** GPER specific agonist (G1; 10 nmol/L), in the presence or absence of eNOS inhibitors L-NAME (1 µmol/L), L-NMMA (1 µmol/L) and of a VEGF-A specific neutralizing antibody (VEGF Ab, 500 ng/ml) on capillary formation by HUVECs. Cells were plated at a density of 4000 cells per well on a matrigel–coated 15-well µ- slides and pretreated with the inhibitors for 30min, subsequently G1 was added. After incubation overnight the capillary formation was assessed microscopically (see Method section 3.2.2). Values represent mean±SEM, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

Discussion

Physiologically, the formation of new capillaries is tightly controlled, if not, the pathological processes, such as tumor formation, inflammatory diseases, gynecological diseases and diabetic retinopathy may occur permanently. VEGF and NO are both effective and critically induce angiogenesis [188, 191, 192], by promoting EC survival, proliferation and migration [184, 213-218, 315]. Moreover,

VEGF and NO interact reciprocally and stimulate each other. NO is both, a downstream and an upstream mediator of VEGF-mediated capillary formation. VEGF stimulates PI3K/ Akt signaling, leading to the activation of eNOS and subsequently to the generation of NO. Under normoxic conditions, NO induces VEGF expression [188], thereby increasing EC cell proliferation [215, 216] and stimulating angiogenesis. Furthermore, VEGF and NO are targets of estrogenic molecules. Treatment with estradiol has been shown to upregulate VEGF expression in uterine tissue, vascular tissue [316-318] and breast cancer [208-211]. Moreover, estrogen mediated acceleration of endothelial recovery has been shown to be associated with increased VEGF expression [319]. The stimulation of eNOS is one of the best described examples for rapid estrogenic actions, which subsequently enhances NO production and thereby induces endothelial-dependent vasodilation [82]. More importantly, it has also been shown that VEGF and NO are essential mediators of estrogen-induced angiogenesis [99]. Studies further suggest that GPER-mediated vasodilation is in part dependent on endothelium-derived NO, because L-NAME, an eNOS inhibitor, inhibited G1-induced relaxation in rat aorta, carotid, mesenteric and porcine coronary arteries [108, 116, 117]. Hence, we assessed whether GPER activation induces eNOS phosphorylation and increases NO production in HUVECs.

Treatment of HUVECs with the GPER specific agonist G1 increased phosphorylation of eNOS, and this effect was significantly blocked by the GPER specific antagonist G15 and by ER unspecific antagonist ICI (182,780). Moreover, in HUVECs significantly elevated levels of NO were detected upon treatment with G1 and E2. Our observations are consistent with findings of others, who reported that G1 treatment activates eNOS and induces NO production in human ECs [320] and in the left ventricular tissue of rats [321]. These findings suggest that GPER mediated estrogen signaling activates eNOS, thereby resulting in NO production by HUVECs.

Several studies specify the important role of VEGF in embryogenesis. The inactivation of one *VEGF* allele has been shown to result in defective vascularization and embryonic lethality [205, 206]. A Cre-loxP approach to inactivate two specific VEGF isoforms in mice resulted in 50% lethality, while the other 50% exhibited impaired myocardial contractility, heart enlargement and onset of ischemic cardiomyopathy [207]. Furthermore, several tumors, including ER+ breast cancer, show elevated VEGF mRNA levels, and estrogen stimulates VEGF expression [208-

211]. Recently, it has been reported that GPER/ HIF1α signaling induces the expression of VEGF in CAFs and SkBr3 cells, *in vitro* and in a murine xenograft model [250, 322]. Therefore, we assessed whether GPER mediated estrogen signaling stimulates VEGF expression in HUVECs.

In HUVECs, treated for 45 min with G1, we observed a significant increase in VEGF protein, which was specifically blocked by G15. Since treatment time of 45 min is too short for *de novo* protein expression, we further assessed VEGF mRNA expression. The results of qPCR revealed that mRNA levels of VEGF remained unaltered following G1 treatment. Surprisingly, increased VEGF mRNA expression was evident in HUVECs treated with G1 in the presence of G15, suggesting that G1 does not stimulate VEGF mRNA expression. To exclude a possible increase in translation of existing VEGF mRNA upon G1 treatment, we applied the translation inhibitor Cycloheximide CY [323] and observed no change in G1-induced VEGF-A protein expression. This finding indicates that GPER activation does not increase the translation of VEGF mRNA. De Francesco et al. revealed that GPER/ HIF1α signaling stimulated the expression of VEGF mRNA and the activation of *VEGF* promoter reporter in CAFs and SkBr3 cells, by silencing GPER and HIF1α and treating them with $CoCl_2$ [250, 322]. In contrast to De Francesco et al., we applied G1 in HUVECs and detected no change in VEGF mRNA, but an increase in VEGF protein level, which was abrogated with G15, but unaltered with CY. Hence, our findings suggest that GPER activation does not change the expression or translation of VEGF mRNA, but promotes the secretion of VEGF protein from intracellular vesicles in HUVECs. Ovarian cancers have been reported to secrete VEGF, serving as a biomarker of increased risk for metastasis [324] and blockade of VEGF secretion has been shown to inhibit angiogenesis *in vivo* [325]. These studies support our contention that G1 induces VEGF secretion in HUVECs, however, more in depth experiments are necessary to confirm this possibility, including the assessment of VEGF levels in the supernatant of G1-treated HUVECs. Moreover, the role of VEGF-A in mediating the long term vasculogenic/ capillary inducing effects of G1 via GPER activation remains to be further investigated.

Angiogenesis is mediated by VEGF and NO [188, 191, 192], furthermore, both molecules have been shown to be essential for estrogen-induced angiogenesis [99].

Previously, we demonstrated that GPER activation induces vasculogenesis, hence, in the present study we assessed whether GPER-mediated capillary formation by HUVECs is mediated by VEGF and NO.

To determine the role of NO and VEGF, we applied eNOS inhibitors L-NAME and L-NMMA and a VEGF specific neutralizing antibody VEGF Ab. G1-induced vasculogenesis in HUVECs was significantly inhibited by the eNOS inhibitors L-NAME, L-NMMA and by VEGF Ab. Our observation is consistent with the findings of others, showing that L-NAME and L-NMMA blocked substance P-induced angiogenesis of ECs [217] and that eNOS knock out mice have impaired morphogenesis and decreased stabilization of angiogenic vessels [219]. Furthermore, monoclonal antibodies targeting VEGF are clinically applied and used as anti-angiogenic agents in cancer therapy [212]. More importantly, Lindsey et al. reported that L-NAME inhibited G1-stimulated vasorelaxation in Lewis female rats [295]. In summary, all these studies support our results and the notion that NO and VEGF are important mediators for GPER-induced vasculogenesis. We propose that GPER mediated estrogen signaling triggers eNOS activation, which subsequently induces NO production and the secretion of VEGF protein. These factors in concert with each other induce capillary formation of ECs and may accelerate endothelial recovery.

4.6 BIAS Signaling of GPER in Endothelial Cells

Objective

GPCR internalization was long believed to be responsible for the termination of its signaling, however recent findings provide evidence that internalized GPCRs can actually signal further. This internalized signaling or bias signaling is of high importance for the pharmaceutical industry, as the majority of drugs target GPCRs. Bias signaling is induced upon binding of a specific biased ligand, mostly not the "common" ligand, which induces the classical G-protein signaling cascade. GPER is a GPCR, hence we also wanted to scrutinize if bias signaling occurs upon activation with G1.

Introduction

A classical GPCR perceives different intracellular signaling proteins, which mediate distinct signaling. One class of those signaling proteins are GRKs. GRKs mediate the phosphorylation of GPCR`s serine residues at the C-terminus [32], which leads to the recruitment and binding of scaffold proteins called β-arrestins. These proteins mediate the internalization of GPCR via CCPs. This internalization procedure was long believed to be responsible for the desensitization and impairment of GPCR signaling by recycling or by transporting the GPCR to the proteasomes [33]. However, recent studies suggest that β-arrestins can also transduce signals via multiple effector pathways [34]. Depending on the ligand bound to GPCR, β-arrestin-mediated signaling or "normal" G-protein mediated signaling will be activated, and this is known as functional selectivity or biased signaling [35]. Within the ligand-receptor complex, both could be biased. A biased ligand prefers one signaling pathway over the other, meaning that it either signals via G-proteins or via β-arrestins. Compared to biased ligands, the endogenous ligand triggers a G-protein signaling cascade, which is terminated by receptor internalization and is therefore termed neutral [34]. Recent studies suggest that biased agonists have weaker interaction with GPCRs than endogenous agonists. However, biased agonists are thought to not only bind to the usual binding site at TM5, but to additional residues at TM7 and ECL2. This "minor" binding site might be involved in biased signaling [326]. On the other hand, a biased receptor, to which a ligand (be it biased or endogenous) is binding, only signals via a distinct subset of pathways, which are available to this

class of receptor [34]. Studies do not provide a complete picture and a lot more research is needed to fully understand biased signaling.

GPER is a classical GPCR, hence we wanted to assess whether biased signaling occurs when GPER is activated by G1 (a GPER specific ligand/ agonist) in HUVECs.

Methods

As described in section 3.2.

Results

The protein targeted as a marker for bias signaling was MEK, which is phosphorylated upon GPCR bias signaling. HUVECs were treated with two different concentrations of G1 (10 and 100 nmol/L) for different times (30 min to 12 h). In another experimental setup, HUVECs were treated with increasing concentrations of G1 (10 to 1000 nmol/L) for 30 min; treatment with epidermal growth factor EGF (100 ng/ml) for 30 min was used as the positive control in all three experiments. As shown in Figure 37A, neither 10 nmol/L nor 100 nmol/L G1 induced MEK phosphorylation at any time point, while the positive control EGF significantly induced phosphorylation of MEK. Moreover, higher concentrations of G1 were also unable to induce MEK phosphorylation (Figure 37B). Based on these observations, bias signaling of GPER in HUVECs seems unlikely.

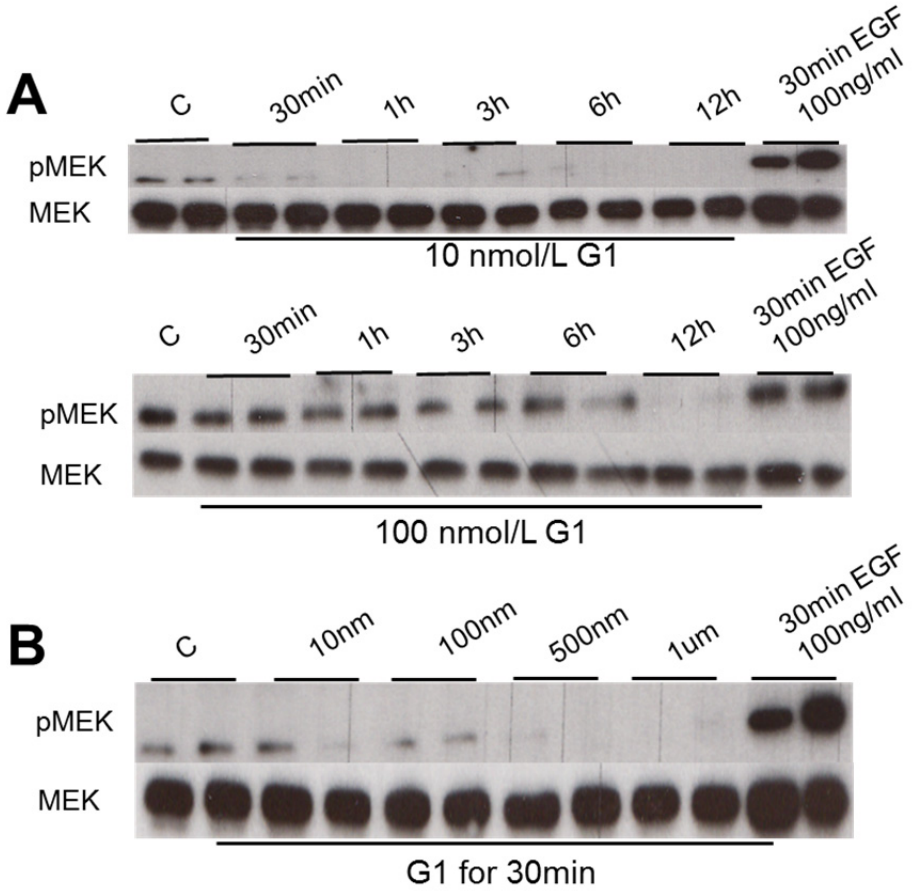

Figure 37. Western Blots showing the effects of **Panel A,** GPER specific agonist (G1; 10 nmol/L and 100 nmol/L) and Epidermal Growth Factor (EGF; 100 ng/ml) on the phosphorylation of MEK. Pre-starved HUVECs were treated with G1 (10 nmol/L or 100 nmol/L) for an increasing time. EGF, a positive control, was applied for 30min. **Panel B,** Western Blot showing the effects of increasing concentrations of G1 on the phosphorylation of MEK. Pre-starved HUVECs were treated with G1 (10-1000 nmol/L) or with EGF (100 ng/ml) for 30 min; EGF was used as a positive control.

Discussion

The key steps of the classical GPCR signaling model involving: 1) ligand binding induced conformational change; 2) the exchange of GDP for GTP, leading to the association of the heterotrimeric G proteins; 3) further production and signaling of second messenger systems; and 4) termination by GRKs that phosphorylate the GPCR and promote binding of β-arrestin and internalization into CCPs; is too simplified and incomplete. Research, conducted over the last ten years, revealed that β-arrestins are not only regulators for the internalization and desensitization of GPCRs, but are adaptor proteins with the ability to signal via many pathways. Pathways, targeted by β-arrestin signaling, include mitogen-activated protein kinase (MAPKs), tyrosine kinase SRC, nuclear factor κB (NF- κB) and PI3K [34].

Activation of SRC and downstream ERK was the first reported example of β-arrestin mediated signaling [327, 328], and was shown to be responsible for preventing apoptosis and increasing mitogenic signals [328]. Subsequently, other studies showed that PAR2 and AT_{1A} receptors signal via β-arrestins to activate ERK [329], MEK1 and ERK1/2 [330]. This β-arrestin-dependent ERK activation was shown to be important for chemotaxis and cytoskeletal rearrangements [331, 332].

Because MAPKs are activated upon β-arrestin signaling and we wanted to assess whether GPER activation induces bias signaling, we studied whether treatment with G1 activates MEK in HUVECs. MEK1 is also known as MAPK/ ERK kinase, and is responsible for controlling cell growth and differentiation.

We observed a time-dependent decrease in phosphorylation of MEK upon treatment with G1 for 30 min to 12 hours. This time frame was chosen because studies suggest that β-arrestin mediated activation is delayed and 100% activation of MEK due to β-arrestin signaling attained after 30 min [333]. Hence, in the next experiment we treated HUVECs for 30 min with increasing concentrations (10–1000 nmol/L) of G1. We detected no increase in pMEK at any concentration of G1 used, suggesting that GPER activation with G1 does not induce β-arrestin-mediated activation of MEK. Based on these observations bias signaling for GPER upon activation with G1 in HUVECs seems unlikely.

Several GPCRs, including β_1- and β_2- adrenergic, AT_{1A}-, μ-opiod, D_2 dopamine- and GPR109A-receptors, have already been described to perform bias signaling and therefore intensively studied [34]. This new research field has immense implications for designing drugs, which target GPCRs. More in depth studies are needed to fully understand bias signaling and to identify GPCRs capable of bias signaling. Although we could not detect activation of MEK upon GPER activation with G1, bias signaling of GPER cannot be completely excluded. Therefore, and because GPER seems to be a good target for new therapies against CVD, more studies in different models are required to elucidate if GPER has the ability to perform bias signaling. Finally, the relevance and explanation for our observation that treatment with G1 downregulated pMEK is unclear and can only be speculated. It is feasible that G1 competes with an endogenous GPCR ligand and blocks its stimulatory effects on MEK. Further studies are required to investigate this possibility.

4.7 GPER Activation inhibits Smooth Muscle Cell Function

Objective

Abnormal growth of SMCs contributes to the vascular remodeling processes associated with CVDs. Estrogens abrogate neointimal thickening in part by inhibiting SMC growth. Although the role of ERα and ERβ in mediating the growth inhibitory effects of estrogen on SMCs is well established, the relative role of the membrane receptor GPER is unclear. Here, we examined effects of GPER activation on the function of SMCs.

Introduction

Endothelial dysfunction [63] and abnormal differentiation and growth of VSMCs [64] is associated with the initiation of atherosclerosis and the progression of CVDs. Findings from several studies provide strong evidence that estrogens induce vasoprotective actions by abrogating the pathological vascular remodeling processes [92] by inhibiting VSMC proliferation *in vivo* in carotid arteries [228, 229] and VSMC migration and proliferation *in vitro* [105, 106]. Additionally, it has been shown that treatment with estrogens decreases myointimal hyperplasia following aortic allografting [88] and in balloon-injured arteries [87]. Because antiproliferative effects of E2 are not lost in VSMCs of ERα and ERβ double knockout mice [123], a potential role for GPER mediating these effects was suggested. Hence, in this study we investigated the effects of GPER on SMC function. We addressed this question by assessing cell proliferation and migration of Human Coronary Aortic Smooth Muscle Cells (HCASMCs).

Methods

As described in section 3.2.

Results

4.7.1 GPER Activation reduces Proliferation

PDGF (20 ng/ml) was employed to induce SMC proliferation, whereas treatment with GPER specific agonist G1 (250 nmol/L), 17β-estradiol E2 (250 nmol/L), GPER specific antagonist (G15; 1 μmol/L), ER-unspecific antagonist ICI 182,780 (ICI; 1 μmol/L) and ERα specific antagonist (MPP; 1 μmol/L) was used to dissect the role of

GPER in mediating SMC growth. As shown in Figure 38A, G1 decreased PDGF-stimulated cell proliferation from 100±2.5% to 67±2.7% ($p<0.05$ relative to control). This inhibitory effect of G1 was significantly reversed in the presence of the GPER specific antagonist G15, the ER unspecific antagonist ICI 182,780 or the ERα specific antagonist MPP from 67±2.7% to 89±4.3%, 93±5.7% and 95±1.6%, respectively ($p<0.05$ relative to G1 treated HCASMCs). Similar results were obtained by E2, which reduced PDGF-induced cell proliferation from 100±5.7% to 69±3%. Moreover, the ER antagonists G15, ICI and MPP abrogated the inhibitory effect of E2 from 69±3% to 92±7.8%, 133±9.4% and 120±8.4%, respectively ($p<0.05$ relative to E2 treated HCASMCs, Figure 38B).

Next, we silenced GPER in HCASMCs, by transfecting them with GPER specific siRNA (50 nmol/L). As shown in Figure 39A, siRNA significantly reduced GPER protein expression by 64% ($p<0.05$ compared to scrambled control). Moreover, silencing of GPER attenuated the inhibitory effect of G1 (250 nmol/L) on PDGF-stimulated cell proliferation from 166±5.1% to 156±12.9% compared to GPER silenced control (Figure 26B). In contrast to HCASMCs treated with GPER siRNA, G1 decreased cell number in SMCs treated with scrambled siRNA from 100±5.3% to 72±4.3% ($p<0.05$ relative to scrambled control, Figure 39B). These findings suggest that GPER activation induces anti-mitogenic effects on HCASMCs. Although inhibition of SMC growth by G1 suggests that the effects are specifically due to GPER activation, however, the finding that G1 effects were reversed by the ERα specific antagonist MPP suggests that the effects of GPER on SMC growth are in part dependent of a cross reaction with ERα.

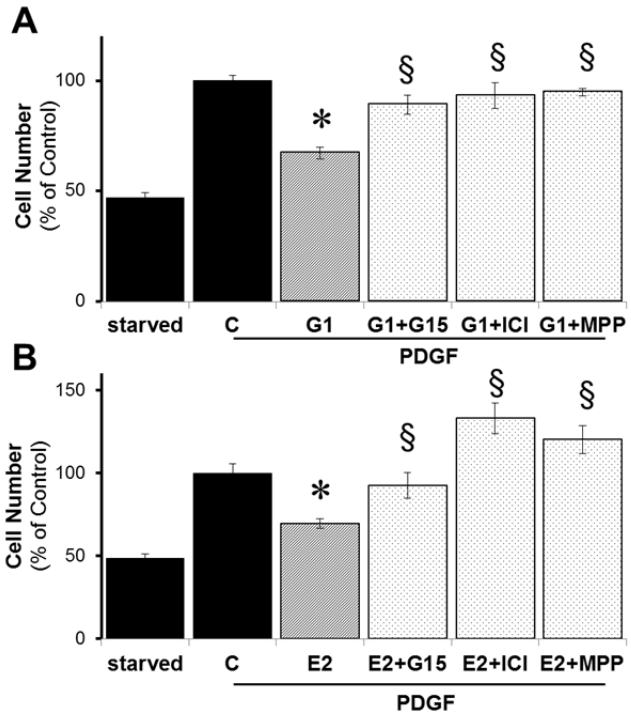

Figure 38. Bar graphs showing the effects of GPER activation on cell proliferation in HCASMCs. Cell proliferation was induced by PDGF (20 ng/ml) and the cells were treated with or without **Panel A)** GPER specific agonist (G1; 250 nmol/L), **Panel B)** estradiol (E2; 250 nmol/L) and in presence or absence of GPER specific antagonist (G15; 1 μmol/L), ER-unspecific antagonist ICI 182,780 (ICI; 1 μmol/L) and ERα specific antagonist (MPP; 1 μmol/L). HCASMCs were plated 80 000 cells/ well in a 12-well plate and grown for 24 h, followed by serum-starving overnight, subsequently cells were pre-treated for 30 min with antagonist, followed by adding the agonists, all in the presence of PDGF (20 ng/ml). Treatment was renewed every 48 h and on day7 cell counting was performed using the Coulter Counter. Values represent mean±SEM, n=5, *P<0.05 relative to control, §P<0.05 relative to G1 or E2 treated HCASMCs, using ANOVA test.

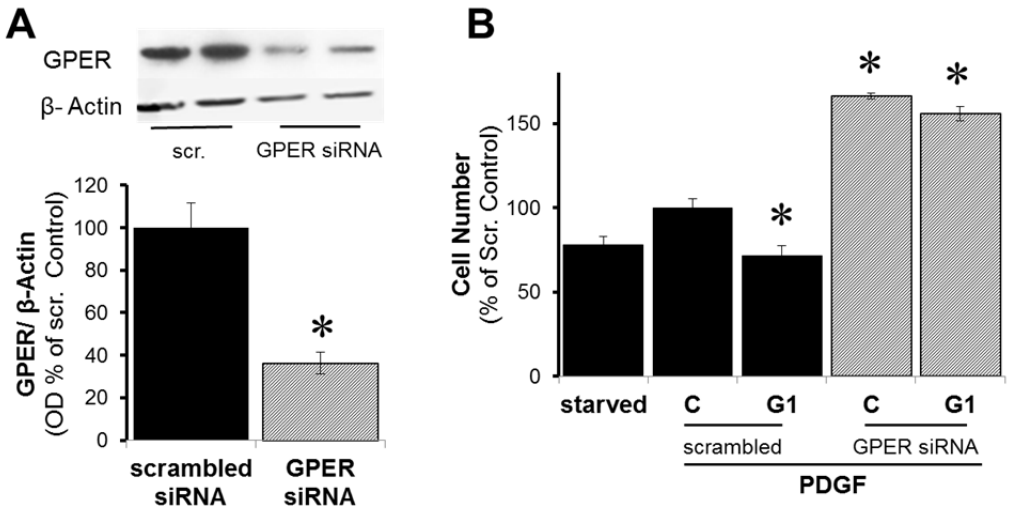

Figure 39. Panel A, Bar graphs and representative Western Blot, showing the efficacy of GPER silencing in HCASMCs using the transfection with scrambled siRNA (50 nmol/L) and GPER siRNA (50 nmol/L). Values represent mean±SEM, *P<0.05 relative to scrambled control using ANOVA test. **Panel B,** Bar graphs depicts the effects of GPER specific agonist (G1; 250 nmol/L) on cell proliferation in HCASMCs, transfected with GPER siRNA or scrambled siRNA. HCASMCs were plated 80 000 cells/ well in a 12-well plated and grown for 24h, followed by serum-starving overnight, subsequently the cells were transfected with siRNA and after 9 h treated for 30 min with G1, in presence of PDGF (20 ng/ml). Treatment was renewed 2x the following 48h. On day3 cell count was performed using the Coulter Counter. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test.

4.7.2 **GPER Activation reduces Migration**

Migration of SMCs from media to neointima leads to vascular remodeling and neointima formation following vascular injury. Therefore, it is important to investigate and identify mechanisms that can prevent increased migration of SMCs. Here we studied the effect of GPER activation by performing a wound closure assay using HCASMCs. A monolayer of HCASMCs, stimulated with PDGF (20 ng/ml), was scratched and treated with G1 (250 nmol/L). We observed that G1 reduced wound closure from 100% to 57±4.7% ($p<0.05$ relative to control). Moreover, G1-reduced wound closure was blocked by G15 (1 µmol/L) and ICI 182,780 (1 µmol /L) from 57±4.7% to 115±8.2% and 108±8.2%, respectively ($p<0.05$ relative G1 treated HCASMCs; Figure 40). Our findings suggest that GPER activation reduces the proliferation of HCASMCs and thereby decreases the possibility of vascular remodeling and neointima formation, leading to CVDs.

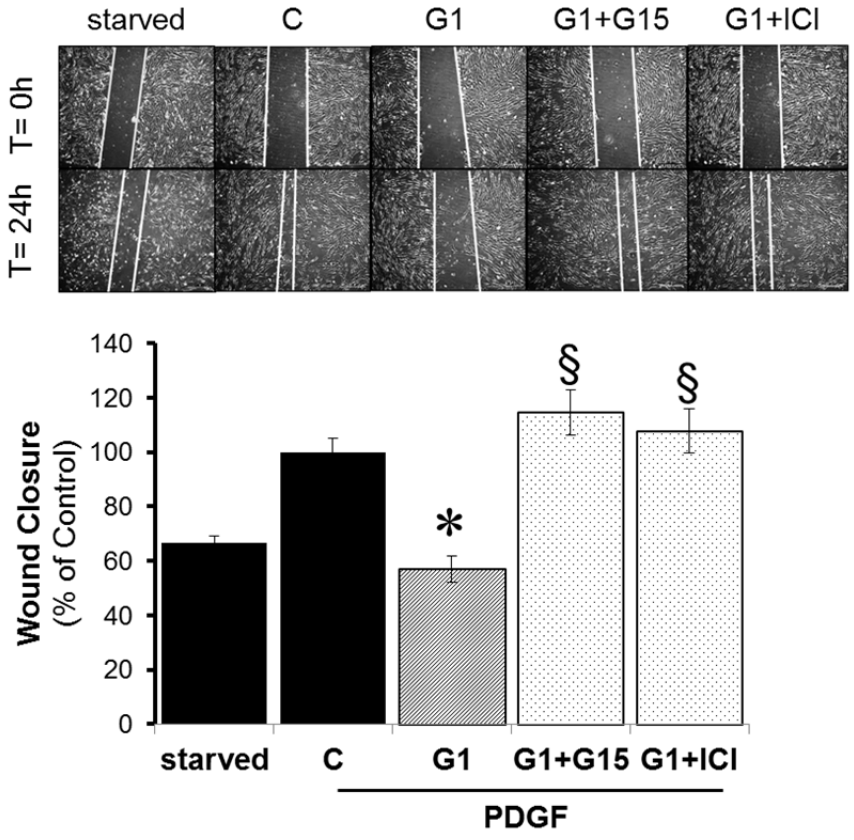

Figure 40. Bar graph and representative photomicrographs showing the effects of GPER activation on wound closure. Wound closure was monitored after scratching a confluent monolayer of HCASMCs, cultured in M231 medium, supplemented with PDGF (20 ng/ml), GPER specific agonist (G1; 250 nmol/L), GPER specific antagonist (G15; 1 µmol/L) or ER-unspecific antagonist ICI 182,780 (ICI; 1 µmol/L). Cells were pretreated with antagonists for 30 min, and subsequently G1 was added and cells were incubated for 24 h. Pictures were taken at time point 0 and 24 h afterwards and analyzed using Excellence Pro software. Values represent mean±SEM, *P<0.05 relative to control, §P<0.05 relative to G1 treated HCASMCs, using ANOVA test using ANOVA test.

Discussion

Although the impact of estrogens on the cardiovascular system is well studied, the mechanisms involved are still unclear. Protective effects of endogenous human estrogens and estrogen replacement therapy against the progression of CVDs have been supported by multiple studies and small clinical trials [78, 79]. The controlled and balanced interaction between ECs and SMCs is essential in preventing CVDs [74]. In this context, acceleration of the endothelial integrity by estrogen contributes to the attenuation of SMC proliferation. This anti-mitogenic effect of estrogens on SMCs is mediated by increasing NO, cGMP [103], cAMP [104] and decreasing CA^{2+} levels [105]. Another protective effect of estrogen is the inhibition of SMCs migration [105, 106], leading to a reduction of myointimal hyperplasia following injury [87, 88].

Vascular SMCs do not only express ERα [230] and ERβ [231], but also GPER [112, 113]. Based on the finding that 17-β-estradiol decreased the proliferation of VSMCs lacking ERα and ERβ i.e. from double knockout mice [123], an important role of GPER in mediating antiproliferative effects by estrogens on SMCs was suggested.

Hence, in this present study we assessed the impact of GPER on SMC function.

We investigated the effect of GPER specific agonist G1 and E2 on the proliferation of HCASMCs and observed a significant reduction in PDGF-induced cell proliferation, which is in line with studies in human and rat VSMCs [110, 113]. These anti-proliferative effects of G1 and E2 on HCASMCs proliferation were reversed by the GPER specific antagonist G15, the ER unspecific inhibitor ICI 182-780 or the ERα specific inhibitor MPP. Because G15 abrogated the inhibitory effects of both G1 and E2, our observation suggests that GPER indeed mediates the antiproliferative effects of estrogens on HCASMCs. In contrast to other groups, who have detected agonistic actions of ICI 182,780, i.e. inducing GPER signaling in breast cancer cell lines [40, 253], we observed a significant abrogation of the antimitogenic effects of G1 by ICI 182,780 in HCASMCs. The differences in the type of cells used might be a possible explanation for these discordant observations. Furthermore, our findings that MPP also reversed the anti-mitogenic effects of G1 and E2, suggests that a crosstalk between GPER and ERα may be necessary to fully elicit the inhibitory signals of estrogen on HCASMCs. Indeed, a crosstalk between GPER and ERα has been

demonstrated in BG-1 ovarian cancer cells, uterine epithelial cells [251, 252] and HUVECs (our observation).

To further elucidate the impact of GPER, HCASMCs were transfected with GPER specific siRNA. Upon silencing of GPER the inhibitory effects of G1 on PDGF-induced HCASMCs proliferation were lost. In contrast, G1 significantly decreased the proliferation of HCASMCS, transfected with scrambled siRNA. Nevertheless, when GPER silenced control was compared to scrambled control we observed a significant increase in the proliferation of HCASMCs. Contrary to our findings, Li et al. showed that the inhibition of human and porcine CASMCs proliferation by G1 treatment was abolished upon GPER silencing [334] and other studies using GPER siRNA observed a significant decrease in G1-stimulated effects in CAFs [258] and SKOV3 cells [259]. A possible explanation for these contrasting outcomes could be the use of different cell types and different GPER siRNAs, although a pooled siRNA was used within this study. GPER is a classical GPCR and possess different subtypes of the heteromeric G proteins Gα/β/γ, which elicit specific signaling. Hence, another plausible explanation for the discordant findings may be that upon GPER silencing, still unknown pro-mitogenic or pro-survivor subunits of GPER have been exposed, thereby leading to this increase in cell number of HCASMCs. However, upon GPER silencing, the inhibitory effects of G1 were lost, indicating the importance of GPER`s role in mediating the inhibitory effects of estrogen on HCASMCs proliferation.

Next, we studied whether GPER activation affects the migration of HCASMCs by using the well established *in vitro* scratch assay [261]. Treatment with G1 significantly decreased the PDGF-induced wound closure of HCASMCs, demonstrating that G1 inhibits the migratory properties of SMCs and mimics the effects of estrogens, as also observed by others [105, 106]. We showed that the inhibitory effects of G1 on wound closure were blocked upon treatment with G15 and ICI 182,780, indicating a role and high specificity of GPER in attenuating HCASMC migration.

Recent studies further underline the importance of GPER`s function in vascular smooth muscle and in the regulation of vascular tone, by inducing the relaxation of vascular arteries [295, 335-337]. Moreover, G1–infusion lowered blood pressure of normotensive [114] and hypertensive rats [108] and G1 treatment improved diastolic dysfunction, cardiac hypertrophy and decreased myocyte size [129]. All these studies emphasize the positive effects of GPER in the cardiovascular system. With regard to

our results in HCASMCs, we suggest a prominent role of GPER in mediating the effects of estrogen on VSMC function, by inhibiting proliferation and migration of SMCs. In conclusion, we propose that GPER might play an essential role in mediating the effects of estrogen in preventing hyperplasia and neointimal thickening by inhibiting SMC function and thereby protecting against vascular remodeling associated with vasoocclusive disorders.

4.8 The PI3K/Akt Pathway mediates Antimitogenic Effects of GPER in Smooth Muscle Cells

Objective

The PI3K/ Akt pathway is a prominent pathway, which regulates multiple cellular functions including metabolism, growth, survival, transcription, protein synthesis and cell proliferation. Moreover, inhibitory effects of estrogens on PI3K/ Akt abrogate SMC proliferation and neointima formation. Hence we wanted to investigate whether there is an association between GPER mediated inhibition of HCASMCs proliferation and PI3K/ Akt signaling.

Introduction

Abnormal differentiation and growth of VSMCs is associated with vascular remodeling, atherosclerosis and the progression of CVDs [64]. Moreover, the PI3K/ Akt pathway plays a central role in controlling vascular cell growth [181]. Studies in Akt deficient mice have reported reduced proliferation, migration and protection against oxidative stress-induced apoptosis of VSMCs [201, 202]. This suggests a potential regulatory role of PI3K/ Akt signaling in response to acute or repetitive injuries to the arterial wall, which contributes to restenosis and atherosclerosis. Interestingly, estrogen abrogates the pathological remodeling processes leading to atherosclerosis [92]; inhibits VSMC proliferation and neointima formation in carotid arteries *in vivo* [228, 229, 338-340]; and the migration and the proliferation of cultured VSMCs [105, 106]. Recent studies suggest that vasoprotective and physiological actions of estrogens are, in part, mediated via rapid, non-nuclear signaling pathways, involving the regulation of specific protein kinases [341, 342]. Ueda et al. reported that PI3K/ Akt is a rapid non-nuclear estrogen signaling target, via which estrogen reduces Akt phosphorylation and is responsible for the inhibition of VSMC proliferation [343]. Based on our findings that GPER-mediated estrogen signaling inhibits the proliferation of HCASMCs, we wanted to investigate whether PI3K/ Akt signaling is the plausible molecular mechanism via which GPER inhibits HCASMCs proliferation.

Methods

As described in section 3.2.

Results

4.8.1 Role of PI3K/ Akt in Proliferation

To examine the role of PI3K/ Akt in SMC proliferation we employed the PI3K-inhibitor LY294002 (LY; 5 µmol/L). Treatment with LY abrogated PDGF (20 ng/ml) - induced phosphorylation of Akt from 100% to 16±1.5% in HCASMCs ($p<0.05$ relative to control, Figure 41A). In agreement with this finding, LY also inhibited PDGF-stimulated cell proliferation from 100% to 61±3%, moreover G1 also inhibited proliferation to a similar extent i.e. 70±2.4% ($p<0.05$ relative to control; Figure 41B). These observations suggest that GPER activation decreases HCASMC proliferation by affecting the PI3K/ Akt pathway.

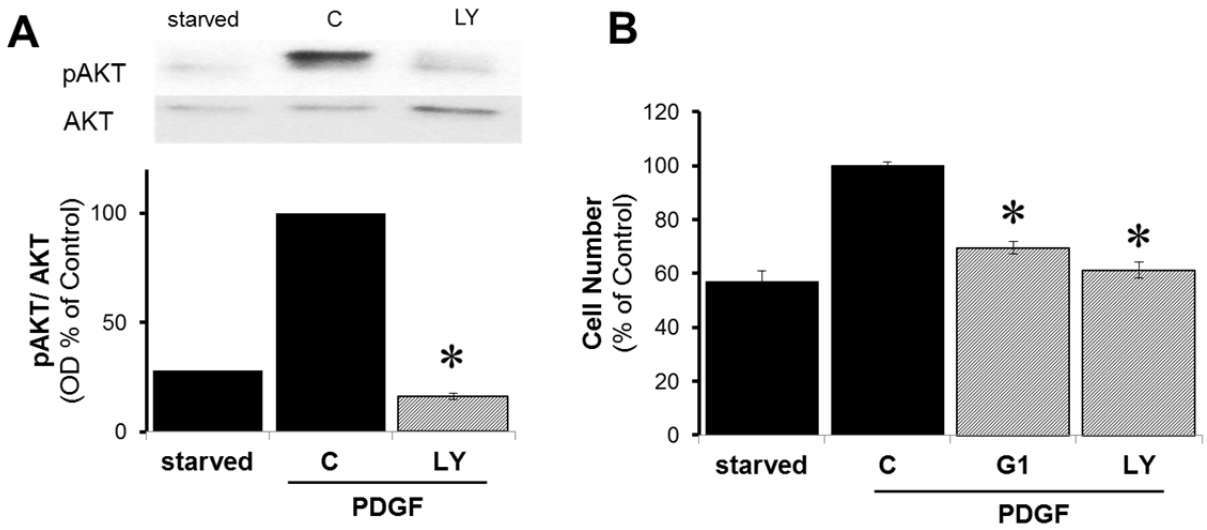

Figure 41. Panel A, Bar graph and representative Western Blot showing the effect of PI3K-inhibitor LY294002 (LY; 5 µmol/L) on PDGF (20 ng/ ml)-induced phosphorylation of Akt. Pre-starved HCASMCs were treated for 30 min with LY in presence of PDGF. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test. **Panel B**, Bar graph depicts the effects of GPER specific agonist (G1; 250 nmol/L) and PI3K-inhibitor LY294002 (LY; 5 µmol/L) on HCASMCs proliferation. Cell proliferation was induced by PDGF (20 ng/ml) and the cells were treated with G1 and LY. HCASMCs were plated 80 000 cells/ well in a 12-well plate and cultured for 24h, subsequently serum-starved overnight and on day0 cells were treated with LY or G1, all in presence of PDGF (20 ng/ml). Treatment was renewed every 48h and on day 7 cell count was performed using the Coulter Counter. Values represent mean±SEM, n=4, *P<0.05 relative to control using ANOVA test.

4.8.2 Inhibition of PI3K/ Akt Pathway

In order to determine if GPER activation affects PI3K/ Akt in HCASMCs, the cells were treated with G1 (100 nmol/L). We observed a significant reduction in PDGF-induced Akt phosphorylation by G1 from 100±9.3% to 65±5.9% ($p<0.05$ relative to control). Moreover, the inhibitory effects of G1 were significantly reversed upon pre-

treatment with G15 (500 nmol/L) and ICI 182,780 (500 nmol/L), from 65±5.9% to 92±7.9% and 102±6.7%, respectively ($p<0.05$ relative to G1 treated HCASMCs, Figure 42A). Furthermore, pre-treatment with MPP (500 nmol/L) reversed G1-inhibited phosphorylation of Akt from 32±6.8% to 105±6.9% ($p<0.05$ relative to G1 treated HCASMCs, Figure 42B). Based on these results we suggest that GPER inhibits HCASMC proliferation via abrogation of the PI3K/ Akt pathway and this effect may in part be dependent on a crosstalk with ERα.

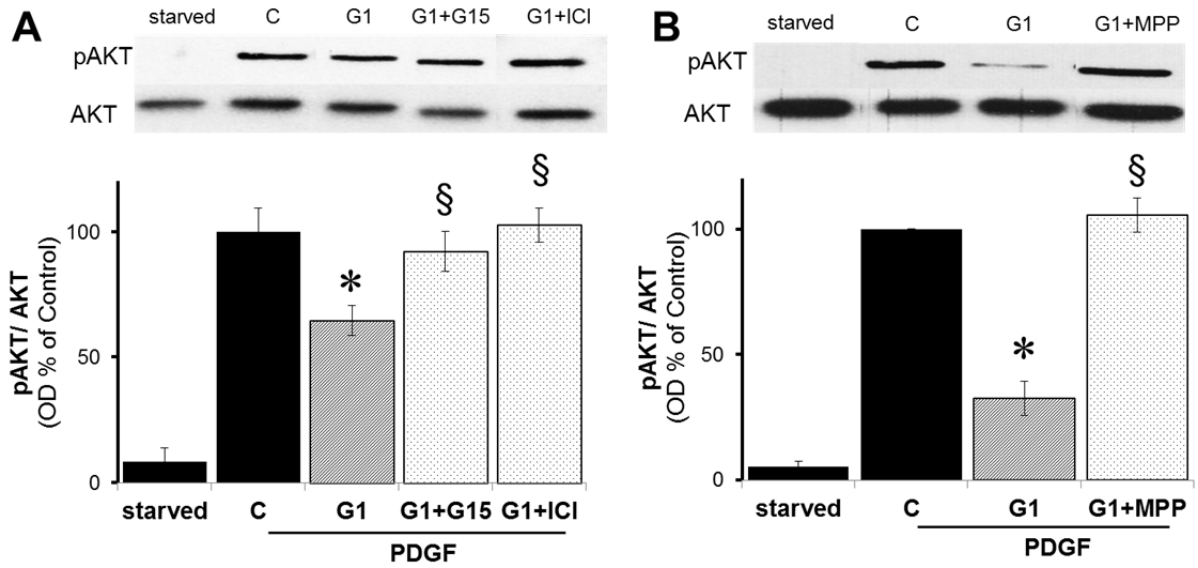

Figure 42. Bar graphs and representative Western Blots showing the effects on Akt phosphorylation. **Panel A**, depicts the effects of GPER specific agonist (G1; 100 nmol/L), GPER specific antagonist (G15; 500 nmol/L), ER-unspecific antagonist ICI 182,780 (ICI; 500 nmol/L), and **Panel B**, depicts the effects of G1 and ERα specific antagonist (MPP; 500 nmol/L). Pre-starved HCASMCs were treated for 30 min with the antagonist, followed by 45 min with G1, all in presence of PDGF (20 ng/ml). Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to G1 using ANOVA test.

Discussion

Abnormal differentiation and growth of VSMCs contributes to the vascular remodeling processes associated with atherosclerosis, coronary artery diseases and restenosis following balloon angioplasty [64]. The highly conserved PI3K/ Akt pathway controls vascular cell viability [180, 181] and importantly regulates the proliferation of SMCs [199, 200]. This notion has been confirmed by *in vivo* studies in Akt1 knockout mice, which showed reduced proliferation, migration and protection against oxidative stress-induced apoptosis of VSMCs [201, 202]. These findings suggest an essential regulatory role of PI3K/ Akt signaling in vascular cell responses to acute or repetitive injuries, which contribute to restenosis and atherosclerosis. Similarly, estrogen treatment has been shown to delay/ abrogate these pathological remodeling

processes, leading to vasooclussive disorders in CVD [92]. Indeed, inhibition of PI3K/ Akt activity with pharmacological inhibitors LY294002 and Wortmannin has been shown to prevent neointimal thickening following balloon injury and PAH [344, 345]. Likewise, estrogen inhibits PI3K/ Akt as well as VSMCs proliferation in carotid arteries *in vivo* [228, 229, 338-340], VSMCs migration and proliferation *in vitro* [105, 106] and abrogates myointimal hyperplasia following injury [87, 88].

It is well documented that estradiol inhibits PI3K/ Akt phosphorylation in SMCs via ERα [343]. However, in addition to the conventional mechanisms, estrogen mediated vasoprotective and physiological responses are also mediated via rapid, non-nuclear signaling pathways, involving the regulation of specific protein kinases [341, 342]. Ueda et al. reported that estrogen mediated inhibition of VSMCs proliferation is mediated by rapid non-nuclear signaling targeting PI3K/ Akt [343]. This contention is confirmed by several other groups as well as by the results of the present study that GPER mediates the inhibitory effects of estrogen on the proliferation of SMCs [110, 113, 334]. Hence, we wanted to investigate whether PI3K/ Akt signaling is the likely molecular mechanism, which mediates the inhibitory actions of GPER on HCASMC proliferation.

Our findings that LY294002, a specific PI3K inhibitor, inhibited PDGF-induced Akt phosphorylation and proliferation of HCASMCs, provides evidence that PI3K/ Akt signaling regulates HCASMCs proliferation, as also reported by others [199, 200]. More importantly, our finding that GPER specific agonist G1 mimicked the effects of LY294002 in reducing the proliferation of HCASMCs, implies that its effects may also be PI3K/ Akt mediated. To elucidate whether GPER activation targets PI3K/ Akt signaling in HCASMCs, we assessed the effect of G1 on Akt phosphorylation. Indeed, treatment with G1 significantly reduced PDGF-stimulated pAkt in HCASMCs. This observation is supported by Li et al. findings, who showed that G1 decreased phosphorylation of Akt and ERK1/2 in human and porcine CASMCs, thereby reducing proliferation in both cell types [334], but these effects could also be unspecific due to the very high G1 concentration (10 µmol/L) used in this study. In contrast to Li et al., the G1 concentration used in our study was several fold lower, 100 nmol/L, and resembles the physiological level of estrogens in premenopausal women [346]. Moreover, the specificity of GPER`s mediated effect on Akt de-phosphorylation was confirmed upon pre-treatment with GPER specific antagonist G15 and ER unspecific

antagonist ICI 182,780, both of which reversed the inhibitory effects of G1 on pAkt in HCASMCs. Our findings indicate that GPER mediated estrogen signaling may stimulate de-phosphorylation of pAKT possibly by activating PP2A, as reported by Ueda et al. [343]. Our observation that MPP, an ERα specific antagonist, abrogated the inhibitory effects of G1 on PDGF-induced pAKT, suggests a potential crosstalk of GPER and ERα is involved in the inhibition of PI3K/ Akt pathway by estrogens in HCASMCs. The importance of ERα in mediating estrogenic inhibitory effects on VSMCs has been reported by other studies, for example ERα knockout mice did not benefit from estrogen`s protective effects on vascular injury [338], while ERβ-deficient mice were still protected [347]. Furthermore, the essential role of GPER in mediating the anti-proliferative effect of estrogens on SMCs was identified by using ERα/ ERβ double knockout mice, which still exhibited E2 mediated reduction of VSMCs proliferation [123]. In summary, our findings suggest that GPER and ERα are important regulators of SMC growth. More importantly, we propose that this GPER/ ERα crosstalk mediates the inhibitory effect of estrogens on PI3K/ Akt signaling which abrogates HCASMC proliferation and initiates vasoprotective actions.

4.9 The ALK1/ SMAD1/5/8 pathway does not mediate Antimitogenic Effects of GPER in Smooth Muscle Cells

Objective

Due to the relevance for the ALK1/ SMAD1/5/8 pathway in mediating GPER dependent growth effects in ECs, we also wanted to investigate the role of this pathway in the anti-mitogenic action of GPER in HCASMCs.

Introduction

The TGFβ superfamily has recently also been shown to play an essential role in vascular homeostasis by influencing VSMC function. TGFβ and BMPs regulate the differentiation and proliferation of SMCs by inducing Matrix-Gla protein (MGP), α-smooth muscle actin (α-SMA) and calponin [168-171, 348]. Although SMCs mainly express the TGFβ type I receptor ALK5, the TGFβ type I receptor ALK1 has been shown to play an important role in modulating phenotype transition in SMCs [164]. Moreover, the interplay between ALK1 and ALK5 seems to be necessary to modulate the effects of TGFβ and BMPs in all vascular cell types, including the SMCs [151, 165]. Additionally, vascular cells have been shown to express other TGFβ type I receptors, such as BMP type I receptors ALK3 and ALK6 [349]. However, contradictory effects of BMP2 on SMC proliferation have been reported. For example, BMP2 is expressed in atherosclerotic lesions and mediates SMC growth [171, 350], whereas findings of Kretzschmar et al. suggest no mitogenic activity of BMP2 [351].

Based on the above findings, we assessed the effect of BMP2 on HCASMCs. Moreover, we investigated whether GPER can modulate ALK1/ SMAD1/5/8 signaling and whether this pathway or the ALK5 pathway contributes to GPER-mediated antimitogenesis in HCASMCs.

Methods

As described in section 3.2.

Results

4.9.1 **Role of BMP2/ ALK5/ ALK1 in Proliferation**

First we determined the role of BMP2 in regulating HCASMCs proliferation by studying its effect in the presence of Noggin, an endogenous antagonist of BMP2 which inhibits the binding of BMP2 and BMP4 to its receptor and their signaling [266].

As shown in Figure 43, treatment with BMP2 (10 ng/ml) increased PDGF-stimulated cell proliferation from 100±2% to 118±7.5% ($p<0.05$ relative to control). BMP2-induced mitogenesis was significantly abrogated by pre-treatment with Noggin (200 ng/ml) from 118±7.5% to 80±3.5% ($p<0.05$ relative to BMP2 treated HCASMCs).

Next we studied whether the ALK1/ SMAD1/5/8 or the ALK5 pathway is involved in BMP2-induced proliferation of HCASMCs by applying SJN2511, a specific ALK5 inhibitor and ALK1Fc, an ALK1 neutralizing antibody. As shown in Figure 43, BMP2-stimulated proliferation was significantly decreased by SJN (200 nmol/L) from 118±7.5% to 61±5.3%, whereas pre-treatment with ALK1Fc (200 ng/ml) had no modulatory effect and was 94±7% ($p<0.05$ relative to BMP2 treated HCASMCs, Figure 30). Our findings provide evidence that BMP2 induces HCASMC proliferation. Moreover, our observation that BMP2 induced proliferation was blocked by Noggin and SJN, but not by ALK1Fc, suggest the involvement of ALK5/ SMAD2/ 3 and not ALK1/ SMAD1/5/8 in mediating the proliferative effects of BMP2 in SMCs.

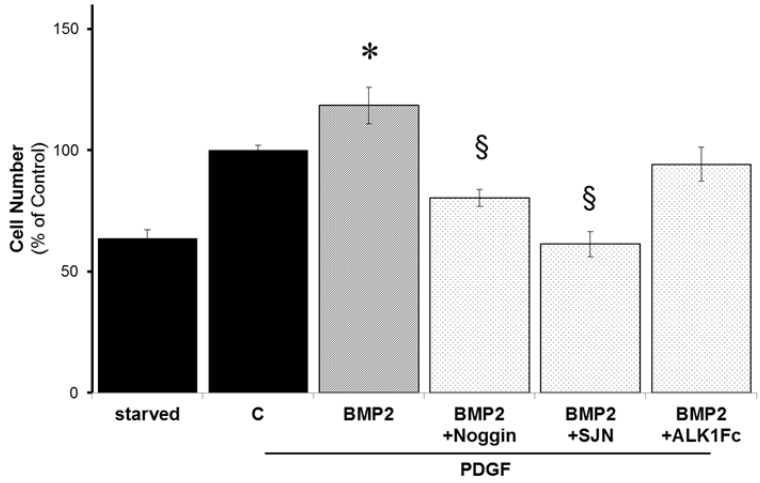

Figure 43. Bar graph showing the effects on cell proliferation in HCASMCs. Cell proliferation was induced by PDGF (20 ng/ml) and the cells treated with or without TGFβ Receptor II specific agonist BMP2 (10 ng/ml), and BMP inhibitor (Noggin, 200 ng/ml) or ALK5 specific antagonist (SJN; 200 nmol/L) or ALK1 specific antagonizing antibody (ALK1Fc; 200 ng/ml). HCASMCs were plated 80 000 cells/ well in a 12-well plate and cultured for 24 h. Followed by serum-starvation overnight, subsequently cells were pre-treated for 30 min with antagonists or inhibitor and then BMP2 was added, all in the presence of PDGF (20 ng/ml). Treatment was renewed every 48 h and on day 7 cell counting was performed using the Coulter Counter. Values represent mean±SEM, n=3, *P<0.05 relative to control, §P<0.05 relative to BMP2 using ANOVA test.

4.9.2 **Activation of ALK1/ SMAD1/5/8 Pathway**

Next we assessed the effects of G1 (250 nmol/L) on the SMAD1/5/8 phosphorylation in HCASMCs. Treatment with G1 significantly increased phosphorylation of SMAD1/5/8 from 100% to 269±7% ($p<0.05$ relative to control, Figure 44A). Moreover, the inhibitory effects of G1 on cell proliferation were not reversed by SJN (200 nmol/L) or by ALK1Fc (200 ng/ml) and changed from 70±2.4% to 75±3.4% and 61±3%, respectively ($p>0.05$ relative to G1 treated HCASMCs, Figure 44B).

In summary, our findings provide evidence that BMP2 induces HCASMC proliferation via ALK5/ SMAD2/3. This finding, together with our observation that activation of GPER with G1 induced SMAD1/5/8 phosphorylation and inhibited HCASMC proliferation and that the antimitogenic effect of G1 was not reversed by ALK5 or ALK1 blockers, suggest that the inhibitory effects of G1 are not mediated via ALK1 nor ALK5, but via some alternative mechanism.

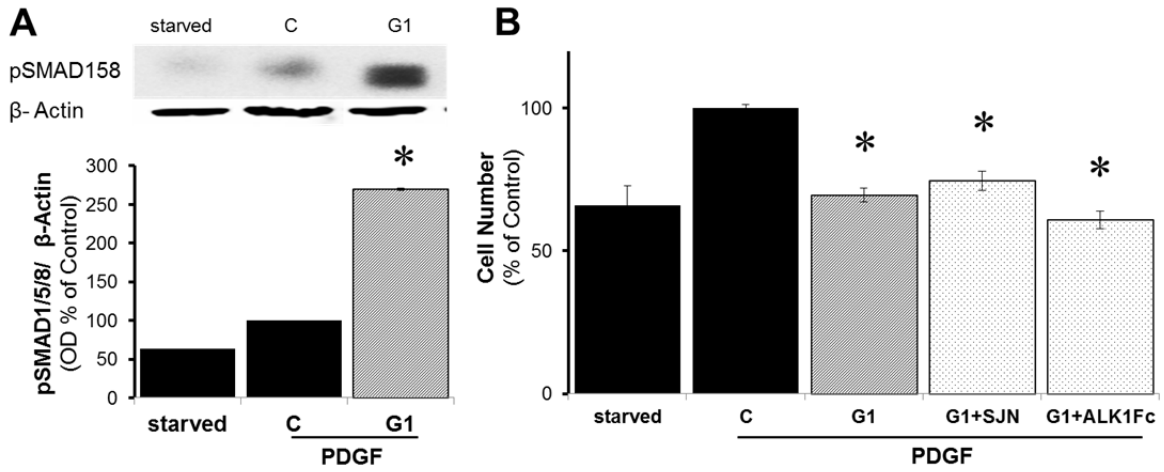

Figure 44. Panel A, Bar graph and representative Western Blot showing the effect of GPER specific agonist (G1; 100 nmol/L) on the phosphorylation of SMAD1/5/8. Pre-starved HCASMCs were treated for 45 min with G1 in the presence of PDGF (20 ng/ml). Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test; **Panel B,** Bar graph showing the effects of GPER specific agonist (G1; 250 nmol/L), in the presence or absence of ALK5 specific antagonist (SJN; 200 nmol/L) and ALK1 specific antagonizing antibody (ALK1Fc; 200 ng/ml) on HCASMC proliferation. Cells were plated 80 000 cells/ well in a 12-well plate and cultured for 24h, followed by serum-starvation overnight. Subsequently cells were pre-treated for 30 min with antagonists and then G1 was added, all in the presence of PDGF (20 ng/ml). Treatment was renewed every 48h, and on day 7 cell counting was performed using the Coulter Counter. Values represent mean±SEM, n=3, *P<0.05 relative to control using ANOVA test.

Discussion

BMPs belong to the TGFβ superfamily, which plays an important role in vascular development and vascular disorder [143]. TGFβ signals via distinct transmembrane receptors known as type I and type II receptors. Activation of these receptors leads to the phosphorylation of different SMADs, which triggers downstream responses, depending on the cell type [262-264]. VSMCs express ALK5, the main TGFβ type I, although presence of ALK1 has also been reported [164], moreover both receptors have to interact, to fully elicit TGFβ and BMP signaling in SMCs [151, 165].

Additionally, the expression of BMP type I receptors ALK3 and ALK6 was demonstrated in vascular cells [349]. However, the effect of the ALK3/ ALK6 ligand BMP2 on SMCs proliferation remains unclear. Some studies state that BMP2 is an important mediator in SMC regulation [171, 350], while others suggest that BMP2 is lacking mitogenic activity [351]. Based on the discordant findings, there is need for ascertaining the role of BMP2 in regulating SMC growth. Hence, in this study we assessed its growth regulatory role in HCASMCs.

We observed a significant increase in HCASMCs number following treatment with BMP2 (10 ng/ml), and pre-treatment with Noggin blocked this effect. Noggin is an endogenous sequestering molecule, which inhibits the binding of BMP2 and BMP4 to their receptors and blocks their downstream signaling [266]. Therefore, the growth stimulatory effect of BMP2 observed in HCASMCs are highly specific and confirm the observations of Willette et al., who reported induction HASMCs proliferation following BMP2 treatment [171].

To study the role of the ALK1 and ALK5 pathways in mediating growth effects in HCASMCs, we used pharmacological inhibitors for ALK1 and ALK5. BMP2 stimulated proliferation of HCASMCs, and this effect was significantly blocked by ALK5- inhibitor SJN2511, but not by ALK1Fc, a neutralizing antibody for ALK1. Our finding suggests that ALK5 signaling plays an important role in inducing SMC proliferation and suggests that BMP2 signaling is not only mediated via ALK3 and ALK6 [265, 282], but potentially involves ALK5. The importance of ALK5 in SMC proliferation was recently emphasized by Tang et al., who reported that TGFβ

induced HASMC differentiation by activating the phosphorylation of SMAD2/3 and SMAD1/5/8 in an ALK5-, but not in an ALK1-dependent manner [348].

Based on the importance of SMAD1/5/8 phosphorylation in HASMC differentiation [348], together with our findings that GPER activation reduces HCASMC proliferation, we assessed whether ALK1- or ALK5- signaling contributes to this effect and whether G1 affects SMAD1/5/8 phosphorylation in HCASMCs. Surprisingly, we found that treatment with G1 significantly induced SMAD1/5/8 phosphorylation and this was accompanied with inhibitory effects on HCASMC proliferation. Based on the fact that SMAD1/5/8 phosphorylation is important for HASMC differentiation [348], together with our finding that BMP2 induces HCASMCs growth; we expected GPER activation would result in inhibition of SMAD1/5/8 phosphorylation, however we observed contrary effects. Moreover, GPER mediated reduction of HCASMC proliferation was not reversed by SJN2511, an inhibitor for ALK5, nor by ALK1Fc, a neutralizing antibody for ALK1. These results, suggest that GPER stimulates ALK1/ SMAD1/5/8 signaling in HCASMCs, but that neither the ALK1- nor the ALK5- pathway contributes to the inhibitory effects of GPER on HCASMCs proliferation.

In summary, our findings provide evidence that BMP2 is an important mitogenic activator, which potentially mediates its effects via ALK5-signaling, moreover the inhibitory effects of GPER on HCASMC proliferation are not mediated via activation of ALK1/ SMAD1/5/8 or the ALK5 pathway.

4.10 Differential Role of GPER Activation on Endothelial and Smooth Muscle Cell Function

Objective

Impaired endothelial function and abnormal differentiation and growth of VSMCs contribute to the onset and progression of vascular remodeling associated with CVDs. The beneficial effects of estrogens on the cardiovascular system are well established; however the mechanisms involved are not completely clear. Treatment with estrogen improves endothelial function and may play a key role in inducing cardiovascular protection, and the controlled and balanced interaction between ECs and SMCs may be essential. Since acceleration of the endothelial recovery by estrogen contributes to the attenuation of SMCs proliferation and migration, we investigated in this study the role of GPER in mediating the protective effects of estrogens and the molecular mechanisms underlying these effects. In this section we provide a summary of the differential effects of estrogen mediated via GPER signaling on HUVECs and HCASMCs.

Introduction

Dysbalance in the homeostasis between endothelium and VSMC activity plays a dominant role in the pathophysiology of vascular remodeling leading to vasoocclusive disorders in CVDs. Endothelial dysfunction, induced by mechanical force or inflammation, initiates biochemical/ molecular signals, which trigger SMCs to abnormally differentiate and proliferate and subsequently lead to neointimal thickening, occlusion of blood vessels, elevated blood flow and high blood pressure, all hallmarks for CVDs [64]. Although many different pathways are involved in mediating these highly complex vascular actions, in this study we solely focused on assessing the role of ALK1/ SMAD1/5/8 and PI3K/ Akt pathway. Both of these pathways are highly conserved and are involved in diverse functions such as growth, adhesion, migration, apoptosis and differentiation of vascular cells [142, 180, 181].

Several studies have demonstrated an important regulatory role of PI3K/ Akt in postnatal blood vessel formation and processes related to angiogenesis [181, 189, 190]. Moreover, PI3K/ Akt has been shown to control the proliferation of cultured SMCs [199, 200]. Role of PI3K/ Akt has also been confirmed by *in vivo* studies, where VSMCs of Akt1 knockout mice showed reduced proliferation, migration and

protection against oxidative stress-induced apoptosis [201, 202]. These findings suggest a regulatory role of PI3K/ Akt signaling in regulating vascular function in responses to acute or repetitive injuries, leading to vasoocclusive disorders.

The ALK1/ SMAD1/5/8/ pathway plays a key role of in embryonic angiogenesis and mutations in ALK1 and SMADs lead to severe defects in embryogenesis and early lethality [176-178]. This pathway is importantly involved in maintaining homeostasis within the vasculature. For example patients with HHT suffer from telangiectiasis, arteriovenous malformation, nose bleeding and gastrointestinal bleeding, show mutations in ALK1 [144]. Although SMCs mainly express ALK5, ALK1 has also been reported to be important for modulating phenotype transition in SMCs [164]. Moreover, interplay between ALK1 and ALK5 seems to be necessary in modulating the effects of its endogenous ligands TGFβ and BMPs on vascular cells [151, 165].

Epidemiological studies suggest a protective role for endogenous estrogens and estrogen replacement therapy on the progression of CVDs [78, 79]. *In vivo* studies provide strong evidence that estrogens induce their vasoprotective actions by inhibiting SMC growth [92] and promoting endothelial function (growth, proliferation, capillary formation) [99, 102, 245]. Estrogen signaling is known to be elicited via two different nuclear receptors ERα, ERβ [78, 102]; moreover the recently identified membrane ER, called GPER, has also been shown to play an active role [38]. GPER is expressed in the vasculature [248], intact arteries [112], ECs [25, 249] and SMCs [112, 113] and has been suggested to mediate important regulatory and protective actions of estrogens within the cardiovascular system.

In the present study we assessed the role of GPER activation on regulating the function of HUVECs and HCASMCs. Moreover, we investigated whether GPER mediates the effects of estrogen on HUVECs and HCASMCs by modulating ALK1/ SMAD1/5/8 and PI3K/ Akt pathways.

Methods

As described in section 3.2.

Results

Our findings provide evidence that GPER activation differentially modulates EC and SMC function. In this context, GPER induces EC and inhibits SMC growth by differentially regulating the PI3K/ Akt and ALK1/ SMAD1/5/8 pathways. Here, we summarize our results from HCASMCs and HUVECs, which highlight the different effects of GPER (described previously under section 4.1-4.9)

Activation of GPER with G1 and E2 abrogated PDGF-induced proliferation of HCASMCs, but induced the formation of new microvessels in HUVECs (Figure 45A/ B). Interestingly, investigation of downstream molecular mechanisms revealed that PI3K/ Akt is importantly regulated in both cell types. In this context, GPER activation differentially modulates PI3K/ Akt in SMCs and ECs. GPER activation by G1 leads to a reduction of AKT phosphorylation in HCASMCs, but induces AKT phosphorylation in HUVECs. Inhibition of PI3K by LY294002, a specific PI3K inhibitor, mimicked the effects of G1 and reduced proliferation of HCASMCs and abrogated G1-stimulated capillary formation by HUVECs. Our findings demonstrate that the ALK1/ SMAD1/5/8 pathway is important in mediating the effects of GPER on capillary formation by HUVECs and does not regulate HCASMC proliferation. Consistent with this notion, we observed that GPER activation with G1 activated ALK1/ SMAD1/5/8 signaling by increasing the phosphorylation of SMAD1/5/8 in both HCASMCs and HUVECs. However, blocking ALK1 abrogated the growth effects of G1 in ECs, but not in SMCs. Taken together, our findings suggest that the capillary-inducing effects of GPER in ECs are ALK1/ SMAD1/5/8 and PI3K/ Akt mediated, whereas the inhibitory effects of GPER in SMCs involve the PI3K/ Akt pathway.

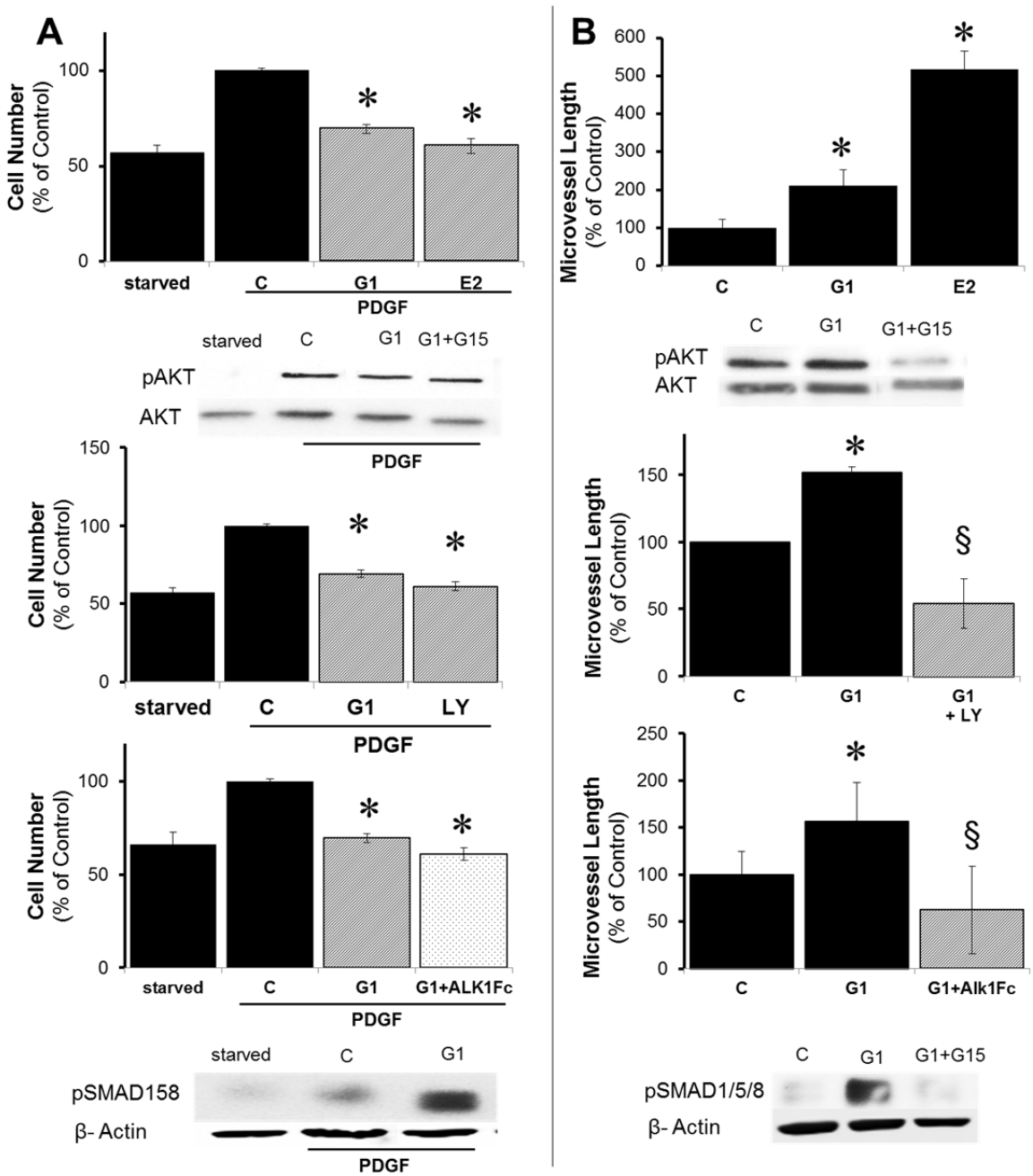

Figure 45. Summary of Bar graphs and representative Western Blots showing the differential effects of GPER specific agonist and estradiol on the function of HCASMCs and HUVECs. **Panel A**, Data from HCASMCs. Bar graphs and representative Western Blots showing the effects of G1 and E2 on cell proliferation and phosphorylation of AKT and SMAD1/5/8. Cell proliferation and phosphorylation of AKT and SMAD1/5/8 was induced by PDGF (20 ng/ml) and the cells were treated with G1, E2, in presence or absence of G15, ALK1Fc or LY294002. Values represent mean±SEM, n=3-5, *P<0.05 relative to control using ANOVA test. **Panel B**, Data from HUVECs. Bar graphs and representative Western Blots showing the effects of G1 and E2 on capillary formation and on phosphorylation of AKT and SMAD1/5/8 of G1 and E2. Cells were treated with G1, E2, in presence or absence of G15, ALK1Fc or LY294002. Values represent mean±SEM, n=5-6, *P<0.05 relative to control using ANOVA test.

Discussion

Although estrogens actively regulate the cardiovascular system, the mechanism(s) involved remain unclear. Protective effects of endogenous estrogens and estrogen replacement therapy against the progression of CVDs are supported by multiple epidemiological studies and small clinical trials [78, 79]. The beneficial actions of estrogen on the endothelium may play an important role in cardiovascular protection [102], furthermore the controlled and balanced crosstalk between ECs and SMCs via autocrine/ paracrine factors may be essential [74]. Indeed, acceleration of the endothelial repair/ growth by estrogen has been shown to attenuate SMC proliferation. The pro-angiogenic effects on EC and the anti-mitogenic effects of estrogens on SMCs are mediated via second messengers like NO, cGMP [103], cAMP [104] and Ca^{2+} [105]. Vascular cells do not only express the nuclear receptors ERα [230] and ERβ [231], but also a membrane receptor, GPER [112, 113], which has been suggested to play an important regulatory role within the cardiovascular system. GPER has been postulated to mediate the anti-proliferative effects of estrogens on SMCs, since the inhibitory effects of 17-β-estradiol on VSMC proliferation were not lost in cells from ERα/ ERβ double knock out mice [123]. Therefore, in the present study we assessed the impact of GPER on HCASMC and HUVEC function by studying cell proliferation and vasculogenesis, respectively.

We observed a significant reduction in PDGF-induced HCASMC proliferation by GPER specific agonist G1 and E2, which is consistent with earlier findings in human and rat VSMCs [110, 113]. Matrigel-based vasculogenesis assay was used to assess the impact of GPER on endothelial function. We observed a significant stimulation of microvessel formation by HUVECs following G1 and E2 treatment, which is consistent with the observations of De Francesco et al. and Baruscotti et al. [100, 250]. In summary, our findings suggest that GPER plays an important role in mediating the differential effects of estradiol on SMC and EC function.

Recently, it has been suggested that estrogen mediated physiological and vasoprotective responses are exhibited via rapid, non-nuclear signaling pathways, involving specific protein kinases [341, 342]. Ueda et al. reported inhibition of VSMCs proliferation by estrogen is mediated via rapid non-nuclear signaling targeting PI3K/ Akt. Indeed, estrogen inhibits Akt phosphorylation and abolishes VSMCs proliferation [343]. Furthermore, GPER mediated estrogen signals are transduced via the PI3K/

Akt pathway, induce survival of MCF-7 cells [306] and migration of renal carcinoma cells [307]. Hence, we wanted to investigate whether PI3K/ Akt signaling is a potential common molecular mechanism, which regulates the effects of GPER on HCASMCs proliferation and capillary formation by HUVECs.

G1 significantly reduced PDGF-stimulated pAkt, implying that GPER activation targets PI3K/ Akt signaling in HCASMCs. Our contention is supported by the findings of Li et al. [334], however in contrast to the high G1 concentration used by Li et al., ours was within the physiological range of estrogen in premenopausal women [346]. The finding that pre-treatment with GPER specific antagonist G15 reversed the inhibitory effects of G1 on pAkt in HCASMCs suggests that the effects of GPER on Akt dephosphorylation are specific. Indeed, treatment with LY294002, a specific PI3K inhibitor, abrogated PDGF-induced HCASMC proliferation, implying that the inhibition of PI3K/ Akt signaling is associated with the reduction of HCASMCs proliferation, as also reported by others [199, 200]. Since G1 mimicked the inhibitory effect of LY294002 on HCASMC proliferation, our findings suggest that estrogen triggers the de-phosphorylation of pAKT, which subsequently inhibits HCASMC proliferation, via GPER.

Contrary to SMCs, in HUVECs we observed a significant increase in Akt phosphorylation followed by G1 treatment, indicating that PI3K/ Akt signaling is activated. This observation is in line with the findings of Guan et al., who reported that G1 treatment specifically increased Akt phosphorylation in renal carcinoma cells and promoted metastasis [307]. The stimulatory effect of G1 on Akt phosphorylation was blocked by G15, indicating that GPER specifically activates PI3K/ Akt signaling. Although the regulatory role of PI3K/ Akt signaling in estrogen-mediated angiogenesis is already proven [100, 240, 241], to our knowledge, we were the first to report the impact of PI3K/ Akt pathway in GPER mediated vasculogenesis. Moreover, G1-stimulated microvessel formation was blocked by LY294002 and reaffirms the role of PI3K/ Akt in mediating the effects of GPER in HUVECs. This is consistent with the findings that LY294002 abrogated G1-induced upregulation of MMP9 in AHCN and OS-RC-2 cells [307], and that wortmannin (another specific PI3K inhibitor) significantly abolished G1-stimulated functional recovery and G1-mediated reduction of infarct size in isolated hearts of Sprague Dawley rats [109]. In summary, the above findings indicate an essential role of the PI3K/ Akt pathway in mediating

the vasoprotective effects of estrogens via GPER. Importantly, via GPER, estrogen differentially regulates PI3K/ Akt in HCASMCs and HUVECs. GPER signaling inhibits proliferation and PI3K/ Akt pathway in HCASMCs most likely by activating PP2A as suggested by Ueda et al. [343], whereas estrogen activates the PI3K/ Akt pathway via GPER and induces vasculogenesis in HUVECs.

The TGFβ superfamily plays an important role in vascular development as well as vascular disorders [143]. TGFβ actively signals via two sets of distinct transmembrane receptors known as type I and type II receptors, which induce further responses by phosphorylating different SMADs (depending on cell type) [262-264]. In VSMCs, ALK5 is the major TGFβ type I receptor expressed, although ALK1 has also been identified [164]. Moreover, both receptors have been shown to interact, to fully elicit TGFβ and BMP signaling in SMCs [151, 165]. ALK1/ SMAD1/5/8 signaling has been shown to regulate HASMC differentiation [348] and endothelial function [160, 267-269]. Furthermore, this pathway has been reported to be essential in the human embryogenesis by regulating angiogenic processes. Mutations in *ALK1* or *SMAD1/5/8* caused early lethality due to vascular abnormalities in murine embryos [176-178]. Several studies have also demonstrated a prominent role of ALK1/ SMAD1/5/8 in modulating postnatal angiogenesis [160, 267-269]. Hence, in this study we assessed whether ALK1/ SMAD1/5/8 signaling is another potential molecular mechanism, which regulates the effects of GPER on HCASMCs proliferation and on capillary formation by HUVECs.

Surprisingly, in HCASMCs treatment with G1 induced SMAD1/5/8 phosphorylation, which has been previously shown to be important for HASMC differentiation [348]. As described in section 4.9.1, we have demonstrated that BMP2 stimulates the proliferation of HCASMCs. Other studies have also reported findings of induction of pSMAD1/5/8 in vascular cells [283] and stimulation of cell proliferation in HASMCs [171] following BMP2 treatment. Since G1 inhibits HCASMC proliferation, we expected it to inhibit SMAD1/5/8 phosphorylation, however we observed contrary effects. Interestingly, GPER mediated inhibition of HCASMC proliferation of HCASMCs was not reversed using ALK1Fc, an ALK1 neutralizing antibody. Based on the above findings, we can conclude that the anti-proliferative effects of GPER on SMCs are not mediated via the ALK1/ SMAD1/5/8 pathway, but rather involve PI3K/ Akt signaling.

In HUVECs treatment with G1 significantly increased SMAD1/5/8 phosphorylation and this effect was significantly inhibited by the GPER specific antagonist G15, implying that GPER specifically activates ALK1/ SMAD1/5/8 signaling. While Matsumoto et al. previously demonstrated the stimulatory effects of estrogens on SMAD1/5/8 phosphorylation [271, 272], to our knowledge, we are the first to demonstrate a regulatory role of GPER in phosphorylating SMAD1/5/8. This contention is further supported by the fact that we were able to block G1-stimulated capillary formation by HUVECs with ALK1Fc, an ALK1 neutralizing antibody. Our observations are consistent with other studies, which demonstrated that ALK1Fc blocks angiogenesis by HMVECs [174] and downregulates the expression of ID-1, a downstream product of ALK1 [149, 174, 273]. In summary, our findings provide evidence that GPER activates ALK1/ SMAD1/5/8 signaling in both HCASMCs and HUVECs. Furthermore, the ALK1/ SMAD1/5/8 pathway does not contribute to the inhibitory effects of GPER on HCASMCs proliferation, but plays an essential role in regulating GPER-induced vasculogenesis by HUVECs.

Based on our findings on HCASMCs and HUVECs, as depicted in Figure 46, we propose that GPER mediates the differential effects of estrogen by inhibiting SMCs activity and inducing/ improving EC function. More importantly, GPER differentially regulates the PI3K/ Akt signaling pathway, i.e. G1 inhibits this pathway in HCASMCs, but activates it HUVECs. The impact of GPER on ALK1/ SMAD1/5/8 signaling is identical in HCASMCs and HUVECs, as G1 stimulated SMAD1/5/8 phosphorylation in both cell types. However, based on our finding that ALK1Fc, an ALK1 neutralizing antibody blocked capillary-inducing effects in HUVECs, but not the anti-proliferative effects in HCASMCs, we propose that ALK1/ SMAD1/5/8 plays a crucial role in GPER-induced capillary formation by ECs and is not involved in GPER-mediated inhibition of SMCs proliferation. In conclusion, GPER may play an important role in mediating the protective effects of estrogen on the cardiovascular system by differentially mediating VSMCs and ECs function. Moreover, agonists for GPER, such as G1, may be of therapeutic relevance in treating CVDs in postmenopausal women, as GPER activity remains unchanged in ageing vessels.

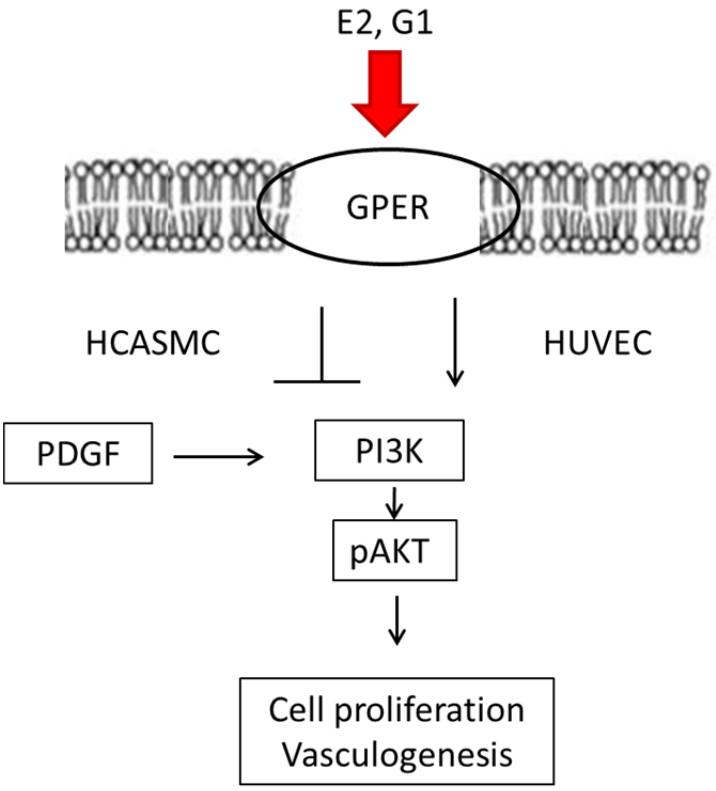

Figure 46: Differential effect of GPER mediated estrogenic signaling on HCASMC and HUVEC. Following GPER activation, the PI3K/ Akt signaling pathway is inhibited in HCASMCs, but stimulated in HUVECs. The differential regulation of this pathway by G1 results in decreased proliferation of HCASMCs, but increased vasculogenesis by HUVECs.

5 Conclusion and Perspectives

Increased life expectancy is also accompanied with ageing associated diseases and highlights the need for research to understand and improve the quality of life and to prevent age-related diseases. Compared to premenopausal women, the prevalence of CVDs is much higher in aging/ postmenopausal women. Moreover, declining estrogen levels correlate with increased incidence of CVDs, suggesting that estrogen protects younger women against CVDs. Indeed, the protective effects of endogenous and therapeutic estrogens against the progression of CVDs are supported by epidemiological/ observational studies, small clinical trials [78, 79]; and selective data of women treated within five years of menopause-onset in the randomized WHI and HERS trials [81, 82]. Estrogens prevent vascular remodeling processes associated with vasoocclusive disorders [92] by improving endothelial growth and function [102] and inhibiting VSMC proliferation [228, 229] [105, 106]. However, estrogens/ HRT can also induce deleterious effects and are implicated in the development of breast and uterine cancer, as well as venous thromboembolic events [136, 137]. Hence, it is essential to pharmacologically separate the beneficial effects on the vasculature from the harmful effects of estrogens on reproductive organs. This may be feasible by selectively targeting ERs with specific agonists, which do not elicit the negative side effects of estrogens. Thus, in depth research is required, to elucidate the complexity of estrogen signaling within the vascular cells and delineate their downstream molecular mechanisms to find an appropriate target. In this study, we investigated the role of GPER mediated estrogen signaling on the function of HUVECs and HCASMCs and the underlying molecular mechanisms.

We demonstrate that GPER activation improves endothelial function by stimulating vasculogenesis, sprouting, migration and proliferation of HUVECs. Our findings provide evidence that GPER can mediate the protective effects of estrogens by accelerating the recovery of damaged endothelium and preventing the vascular remodeling processes leading to CVDs. Importantly, we demonstrate that GPER activation stimulates ALK1/ SMAD1/5/8 signaling by inducing the secretion of BMP2. In summary, the ALK1/ SMAD1/5/8 pathway is partly responsible for GPER-induced capillary formation in HUVECs and ECFCs.

We further discovered that GPER stimulation increases cAMP synthesis in HUVECs, which mediates the beneficial effect of GPER on vasculogenesis. In this context, we revealed that GPER stimulates PKA, which is downstream from cAMP and activates the ALK1/ SMAD1/5/8 signaling cascade, consequently stimulating capillary formation by HUVECs. It has been shown that GPER activation stimulates PI3K/ Akt/ NO/ VEGF signaling, which is partly responsible for GPER-induced capillary formation by HUVECs. However, whether VEGF-A secretion by ECS in response to G1 is responsible or contributes to GPER-induced vasculogenesis, in the long term, remains to be further elucidated and confirmed. Here, we demonstrate that ALK1/ SMAD1/5/8 and PI3K/ Akt signaling crosstalk with each other to promote vasculogenesis.

Apart from endothelial damage and dysfunction, the abnormal differentiation and growth of VSMCs contributes to neointima formation and vascular remodeling processes leading to vasoocclusive disorders. Our findings provide evidence that GPER activation alters HCASMC function by inhibiting their proliferation and migration. Hence, GPER activation abrogates key processes contributing to vascular remodeling leading to vasoocclusive disorders in CVDs. We further investigated the potential molecular mechanisms and identified that GPER activation inhibits PI3K/ Akt signaling, but stimulates ALK1/ SMAD1/5/8 signaling. Moreover, we demonstrate that inhibition of PI3K/ Akt, but not ALK1/ SMAD1/5/8 activation contributes to the reduced HCASMC proliferation by GPER. Furthermore, we provide evidence that a crosstalk between GPER and ERα is responsible for the inhibitory effects of estrogens on PI3K/ Akt signaling, proliferation and migration of HCASMCs.

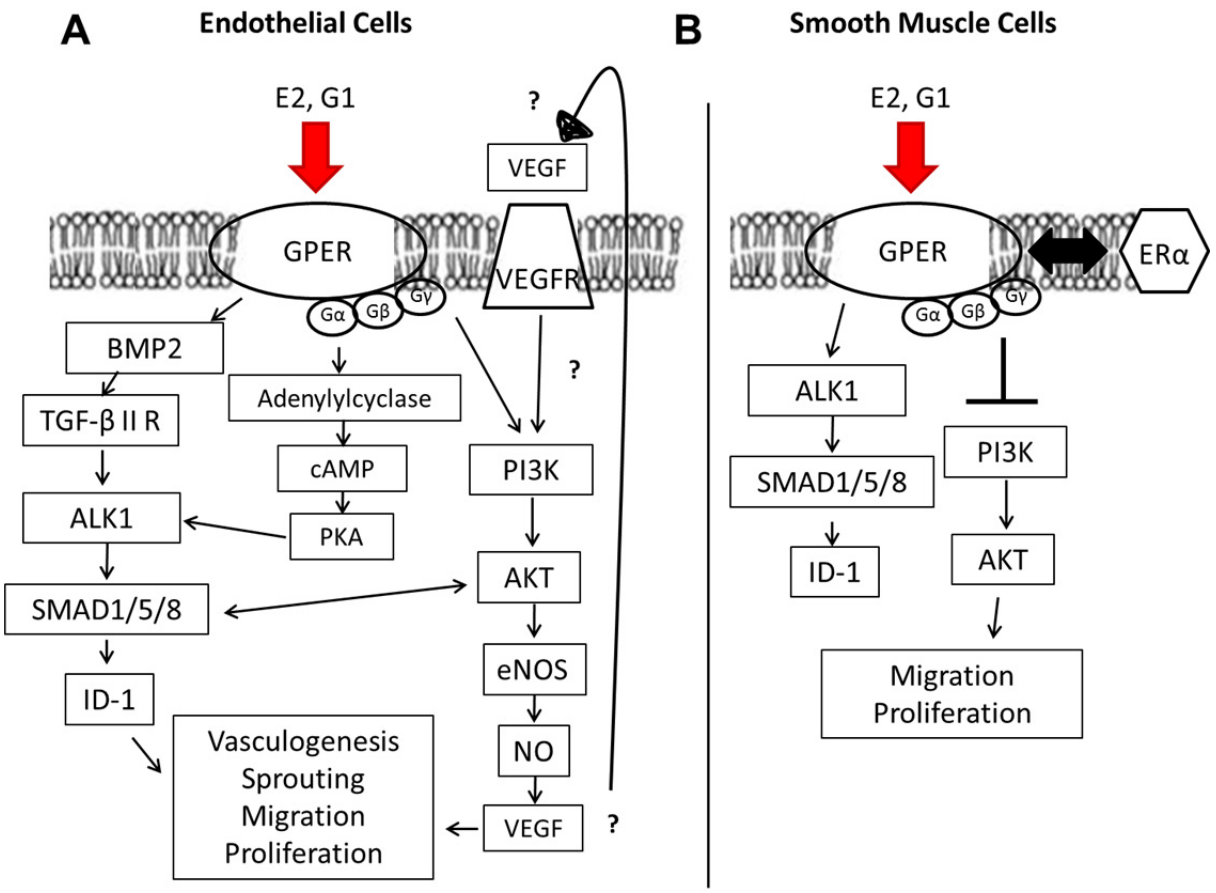

Figure 47: Descriptive Chart of this Study`s Conclusions. **Panel A:** The role of GPER activation on endothelial function and regulating molecular mechanisms, in HUVECs. **Panel B:** The role of GPER activation on SMC function and regulating molecular mechanisms, in HCASMCs.

In summary, our findings demonstrate that GPER plays an important role in mediating the protective effects of estrogen against vasoocclusive disorders associated with CVDs, by improving endothelial function and inhibiting SMC proliferation and migration, as depicted in Figure 47. Our findings suggest that GPER might be an interesting candidate for developing new therapeutic agents to treat CVDs in post-menopausal women, without exhibiting the negative side effects of hormones currently used for replacement therapy. Additionally, GPER function remains unaltered in ageing vessels, in contrast to ERα and ERβ. Therefore GPER ligands/ agonists may be superior to the currently used estrogen therapies in inducing vasoprotective actions. Moreover, GPER agonists do not exhibit the feminizing effects of estrogens and may be considered therefore as a new treatment strategy against CVDs in both genders.

References

1. Tulchinsky, D., et al., *Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy.* Am J Obstet Gynecol, 1972. **112**(8): p. 1095-100.
2. Gruber, C.J., et al., *Production and actions of estrogens.* N Engl J Med, 2002. **346**(5): p. 340-52.
3. Nelson, L.R. and S.E. Bulun, *Estrogen production and action.* J Am Acad Dermatol, 2001. **45**(3 Suppl): p. S116-24.
4. Yager, J.D., *Endogenous estrogens as carcinogens through metabolic activation.* J Natl Cancer Inst Monogr, 2000(27): p. 67-73.
5. Bulun, S.E., et al., *Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance.* J Mol Endocrinol, 2000. **25**(1): p. 35-42.
6. Cignarella, A., M. Kratz, and C. Bolego, *Emerging role of estrogen in the control of cardiometabolic disease.* Trends Pharmacol Sci, 2010. **31**(4): p. 183-9.
7. Ling, S., P. Komesaroff, and K. Sudhir, *Cellular mechanisms underlying the cardiovascular actions of oestrogens.* Clin Sci (Lond), 2006. **111**(2): p. 107-18.
8. Dubey, R.K. and E.K. Jackson, *Cardiovascular protective effects of 17beta-estradiol metabolites.* J Appl Physiol (1985), 2001. **91**(4): p. 1868-83.
9. Zhu, B.T. and A.H. Conney, *Functional role of estrogen metabolism in target cells: review and perspectives.* Carcinogenesis, 1998. **19**(1): p. 1-27.
10. Mooberry, S.L., *New insights into 2-methoxyestradiol, a promising antiangiogenic and antitumor agent.* Curr Opin Oncol, 2003. **15**(6): p. 425-30.
11. Barchiesi, F., et al., *2-Methoxyestradiol, an estradiol metabolite, inhibits neointima formation and smooth muscle cell growth via double blockade of the cell cycle.* Circ Res, 2006. **99**(3): p. 266-74.
12. Dubey, R.K. and E.K. Jackson, *Potential vascular actions of 2-methoxyestradiol.* Trends Endocrinol Metab, 2009. **20**(8): p. 374-9.
13. Greene, G.L., et al., *Sequence and expression of human estrogen receptor complementary DNA.* Science, 1986. **231**(4742): p. 1150-4.
14. Kuiper, G.G., et al., *Cloning of a novel receptor expressed in rat prostate and ovary.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5925-30.
15. Ogawa, S., et al., *The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro.* Biochem Biophys Res Commun, 1998. **243**(1): p. 122-6.
16. Edwards, D.P., *Regulation of signal transduction pathways by estrogen and progesterone.* Annu Rev Physiol, 2005. **67**: p. 335-76.
17. Nilsson, S., et al., *Mechanisms of estrogen action.* Physiol Rev, 2001. **81**(4): p. 1535-65.
18. Saceda, M., et al., *Regulation of the estrogen receptor in MCF-7 cells by estradiol.* Mol Endocrinol, 1988. **2**(12): p. 1157-62.
19. Kaneko, K.J., J.D. Furlow, and J. Gorski, *Involvement of the coding sequence for the estrogen receptor gene in autologous ligand-dependent down-regulation.* Mol Endocrinol, 1993. **7**(7): p. 879-88.
20. Marin-Castano, M.E., et al., *Regulation of estrogen receptors and MMP-2 expression by estrogens in human retinal pigment epithelium.* Invest Ophthalmol Vis Sci, 2003. **44**(1): p. 50-9.
21. Denger, S., et al., *Tissue-specific expression of human ERalpha and ERbeta in the male.* Mol Cell Endocrinol, 2001. **178**(1-2): p. 155-60.

22. Owman, C., et al., *Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues.* Biochem Biophys Res Commun, 1996. **228**(2): p. 285-92.
23. Kvingedal, A.M. and E.B. Smeland, *A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue.* FEBS Lett, 1997. **407**(1): p. 59-62.
24. Carmeci, C., et al., *Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer.* Genomics, 1997. **45**(3): p. 607-17.
25. Takada, Y., et al., *Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress.* Biochem Biophys Res Commun, 1997. **240**(3): p. 737-41.
26. O'Dowd, B.F., et al., *Discovery of three novel G-protein-coupled receptor genes.* Genomics, 1998. **47**(2): p. 310-3.
27. Feng, Y. and P. Gregor, *Cloning of a novel member of the G protein-coupled receptor family related to peptide receptors.* Biochem Biophys Res Commun, 1997. **231**(3): p. 651-4.
28. Venkatakrishnan, A.J., et al., *Molecular signatures of G-protein-coupled receptors.* Nature, 2013. **494**(7436): p. 185-94.
29. Rasmussen, S.G., et al., *Crystal structure of the beta2 adrenergic receptor-Gs protein complex.* Nature, 2011. **477**(7366): p. 549-55.
30. Katritch, V., V. Cherezov, and R.C. Stevens, *Diversity and modularity of G protein-coupled receptor structures.* Trends Pharmacol Sci, 2012. **33**(1): p. 17-27.
31. Tuteja, N., *Signaling through G protein coupled receptors.* Plant Signal Behav, 2009. **4**(10): p. 942-7.
32. Pingret, J.L., E.P. Journet, and D.G. Barker, *Rhizobium nod factor signaling. Evidence for a g protein-mediated transduction mechanism.* Plant Cell, 1998. **10**(5): p. 659-72.
33. Moore, C.A., S.K. Milano, and J.L. Benovic, *Regulation of receptor trafficking by GRKs and arrestins.* Annu Rev Physiol, 2007. **69**: p. 451-82.
34. Rajagopal, S., K. Rajagopal, and R.J. Lefkowitz, *Teaching old receptors new tricks: biasing seven-transmembrane receptors.* Nat Rev Drug Discov, 2010. **9**(5): p. 373-86.
35. Kenakin, T., *Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors.* Trends Pharmacol Sci, 2007. **28**(8): p. 407-15.
36. Gilman, A.G., *G proteins: transducers of receptor-generated signals.* Annu Rev Biochem, 1987. **56**: p. 615-49.
37. Ford, C.E., et al., *Molecular basis for interactions of G protein betagamma subunits with effectors.* Science, 1998. **280**(5367): p. 1271-4.
38. Filardo, E.J., et al., *Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF.* Mol Endocrinol, 2000. **14**(10): p. 1649-60.
39. Filardo, E.J., et al., *Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis.* Mol Endocrinol, 2002. **16**(1): p. 70-84.
40. Revankar, C.M., et al., *A transmembrane intracellular estrogen receptor mediates rapid cell signaling.* Science, 2005. **307**(5715): p. 1625-30.

41. Thomas, P., et al., *Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells.* Endocrinology, 2005. **146**(2): p. 624-32.
42. Prossnitz, E.R. and M. Barton, *The G-protein-coupled estrogen receptor GPER in health and disease.* Nat Rev Endocrinol, 2011. **7**(12): p. 715-26.
43. Palczewski, K., et al., *Crystal structure of rhodopsin: A G protein-coupled receptor.* Science, 2000. **289**(5480): p. 739-45.
44. Hurst, D.P., et al., *A lipid pathway for ligand binding is necessary for a cannabinoid G protein-coupled receptor.* J Biol Chem, 2010. **285**(23): p. 17954-64.
45. Bologa, C.G., et al., *Virtual and biomolecular screening converge on a selective agonist for GPR30.* Nat Chem Biol, 2006. **2**(4): p. 207-12.
46. Dennis, M.K., et al., *In vivo effects of a GPR30 antagonist.* Nat Chem Biol, 2009. **5**(6): p. 421-7.
47. Faulds, M.H., et al., *The diversity of sex steroid action: regulation of metabolism by estrogen signaling.* J Endocrinol, 2012. **212**(1): p. 3-12.
48. Watts, V.J., et al., *Dopamine D2 receptor-induced heterologous sensitization of adenylyl cyclase requires Galphas: characterization of Galphas-insensitive mutants of adenylyl cyclase V.* Mol Pharmacol, 2001. **60**(6): p. 1168-72.
49. Pang, Y., J. Dong, and P. Thomas, *Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest.* Endocrinology, 2008. **149**(7): p. 3410-26.
50. Otto, C., et al., *G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol.* Endocrinology, 2008. **149**(10): p. 4846-56.
51. Filardo, E.J. and P. Thomas, *Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology.* Endocrinology, 2012. **153**(7): p. 2953-62.
52. Lundstrom, K., *An overview on GPCRs and drug discovery: structure-based drug design and structural biology on GPCRs.* Methods Mol Biol, 2009. **552**: p. 51-66.
53. Beato, M., P. Herrlich, and G. Schutz, *Steroid hormone receptors: many actors in search of a plot.* Cell, 1995. **83**(6): p. 851-7.
54. Hall, J.M., J.F. Couse, and K.S. Korach, *The multifaceted mechanisms of estradiol and estrogen receptor signaling.* J Biol Chem, 2001. **276**(40): p. 36869-72.
55. Muramatsu, M. and S. Inoue, *Estrogen receptors: how do they control reproductive and nonreproductive functions?* Biochem Biophys Res Commun, 2000. **270**(1): p. 1-10.
56. Kato, S., *Estrogen receptor-mediated cross-talk with growth factor signaling pathways.* Breast Cancer, 2001. **8**(1): p. 3-9.
57. Chambliss, K.L., et al., *Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae.* Circ Res, 2000. **87**(11): p. E44-52.
58. Migliaccio, A., et al., *Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells.* EMBO J, 1996. **15**(6): p. 1292-300.
59. Chen, Z., et al., *Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen.* J Clin Invest, 1999. **103**(3): p. 401-6.

60. Coleman, K.M. and C.L. Smith, *Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors.* Front Biosci, 2001. **6**: p. D1379-91.
61. Kampa, M., et al., *The estrogen receptor: two or more molecules, multiple variants, diverse localizations, signaling and functions. Are we undergoing a paradigm-shift as regards their significance in breast cancer?* Hormones (Athens), 2013. **12**(1): p. 69-85.
62. Murray, C.J. and A.D. Lopez, *Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study.* Lancet, 1997. **349**(9064): p. 1498-504.
63. Ross, R., *The pathogenesis of atherosclerosis: a perspective for the 1990s.* Nature, 1993. **362**(6423): p. 801-9.
64. Owens, G.K., *Regulation of differentiation of vascular smooth muscle cells.* Physiol Rev, 1995. **75**(3): p. 487-517.
65. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
66. Losordo, D.W., J.M. Isner, and L.J. Diaz-Sandoval, *Endothelial recovery: the next target in restenosis prevention.* Circulation, 2003. **107**(21): p. 2635-7.
67. Carmeliet, P., *Angiogenesis in life, disease and medicine.* Nature, 2005. **438**(7070): p. 932-6.
68. Carmeliet, P., *Mechanisms of angiogenesis and arteriogenesis.* Nat Med, 2000. **6**(4): p. 389-95.
69. Flamme, I., T. Frolich, and W. Risau, *Molecular mechanisms of vasculogenesis and embryonic angiogenesis.* J Cell Physiol, 1997. **173**(2): p. 206-10.
70. Simons, M., *Angiogenesis: where do we stand now?* Circulation, 2005. **111**(12): p. 1556-66.
71. Skalak, T.C., *Angiogenesis and microvascular remodeling: a brief history and future roadmap.* Microcirculation, 2005. **12**(1): p. 47-58.
72. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
73. Adair, T.H. and J.P. Montani, *Angiogenesis*. 2010, San Rafael CA: 2010 by Morgan & Claypool Life Sciences.
74. Hungerford, J.E., et al., *Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers.* Dev Biol, 1996. **178**(2): p. 375-92.
75. Umemura, T. and Y. Higashi, *Endothelial progenitor cells: therapeutic target for cardiovascular diseases.* J Pharmacol Sci, 2008. **108**(1): p. 1-6.
76. Sullivan, J.M. and L.P. Fowlkes, *The clinical aspects of estrogen and the cardiovascular system.* Obstet Gynecol, 1996. **87**(2 Suppl): p. 36S-43S.
77. Senoz, S., et al., *Estrogen deprivation, rather than age, is responsible for the poor lipid profile and carbohydrate metabolism in women.* Maturitas, 1996. **25**(2): p. 107-14.
78. Dubey, R.K. and E.K. Jackson, *Estrogen-induced cardiorenal protection: potential cellular, biochemical, and molecular mechanisms.* Am J Physiol Renal Physiol, 2001. **280**(3): p. F365-88.
79. Dubey, R.K., et al., *Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen.* Cardiovasc Res, 2005. **66**(2): p. 295-306.
80. Grodstein, F., et al., *Postmenopausal hormone therapy and mortality.* N Engl J Med, 1997. **336**(25): p. 1769-75.

81. Clarkson, T.B., G.C. Melendez, and S.E. Appt, *Timing hypothesis for postmenopausal hormone therapy: its origin, current status, and future.* Menopause, 2013. **20**(3): p. 342-53.
82. Miller, V.M. and S.P. Duckles, *Vascular actions of estrogens: functional implications.* Pharmacol Rev, 2008. **60**(2): p. 210-41.
83. Mikkola, T.S. and T.B. Clarkson, *Estrogen replacement therapy, atherosclerosis, and vascular function.* Cardiovasc Res, 2002. **53**(3): p. 605-19.
84. Mozaffarian, D., et al., *Heart disease and stroke statistics--2015 update: a report from the American Heart Association.* Circulation, 2015. **131**(4): p. e29-322.
85. Foegh, M.L., et al., *Estradiol protects against experimental cardiac transplant atherosclerosis.* Transplant Proc, 1987. **19**(4 Suppl 5): p. 90-5.
86. Cheng, L.P., et al., *Inhibition of myointimal hyperplasia and macrophage infiltration by estradiol in aorta allografts.* Transplantation, 1991. **52**(6): p. 967-72.
87. Foegh, M.L., et al., *Estradiol inhibition of arterial neointimal hyperplasia after balloon injury.* J Vasc Surg, 1994. **19**(4): p. 722-6.
88. Jacobsson, J., et al., *Effect of estradiol on accelerated atherosclerosis in rabbit heterotopic aortic allografts.* J Heart Lung Transplant, 1992. **11**(6): p. 1188-93.
89. Chen, S.J., et al., *Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery.* Circulation, 1996. **93**(3): p. 577-84.
90. Oparil, S., et al., *Sexually dimorphic response of the balloon-injured rat carotid artery to hormone treatment.* Circulation, 1997. **95**(5): p. 1301-7.
91. Bakir, S., et al., *Estrogen-induced vasoprotection is estrogen receptor dependent: evidence from the balloon-injured rat carotid artery model.* Circulation, 2000. **101**(20): p. 2342-4.
92. Bowling, M.R., et al., *Estrogen effects on vascular inflammation are age dependent: role of estrogen receptors.* Arterioscler Thromb Vasc Biol, 2014. **34**(7): p. 1477-85.
93. Murphy, E., *Estrogen signaling and cardiovascular disease.* Circ Res, 2011. **109**(6): p. 687-96.
94. Geraldes, P., et al., *Estrogen regulation of endothelial and smooth muscle cell migration and proliferation: role of p38 and p42/44 mitogen-activated protein kinase.* Arterioscler Thromb Vasc Biol, 2002. **22**(10): p. 1585-90.
95. Van Belle, E., et al., *Endothelial regrowth after arterial injury: from vascular repair to therapeutics.* Cardiovasc Res, 1998. **38**(1): p. 54-68.
96. Filipe, C., et al., *Estradiol accelerates endothelial healing through the retrograde commitment of uninjured endothelium.* Am J Physiol Heart Circ Physiol, 2008. **294**(6): p. H2822-30.
97. Strehlow, K., et al., *Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation.* Circulation, 2003. **107**(24): p. 3059-65.
98. Iwakura, A., et al., *Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury.* Circulation, 2003. **108**(25): p. 3115-21.
99. Losordo, D.W. and J.M. Isner, *Estrogen and angiogenesis: A review.* Arterioscler Thromb Vasc Biol, 2001. **21**(1): p. 6-12.

100. Baruscotti, I., et al., *Estradiol stimulates capillary formation by human endothelial progenitor cells: role of estrogen receptor-{alpha}/{beta}, heme oxygenase 1, and tyrosine kinase.* Hypertension, 2010. **56**(3): p. 397-404.
101. Rubanyi, G.M., A. Johns, and K. Kauser, *Effect of estrogen on endothelial function and angiogenesis.* Vascul Pharmacol, 2002. **38**(2): p. 89-98.
102. Mendelsohn, M.E. and R.H. Karas, *The protective effects of estrogen on the cardiovascular system.* N Engl J Med, 1999. **340**(23): p. 1801-11.
103. Cornwell, T.L., et al., *Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP.* Am J Physiol, 1994. **267**(5 Pt 1): p. C1405-13.
104. Dubey, R.K., et al., *Estradiol inhibits smooth muscle cell growth in part by activating the cAMP-adenosine pathway.* Hypertension, 2000. **35**(1 Pt 2): p. 262-6.
105. Bhalla, R.C., et al., *Estrogen reduces proliferation and agonist-induced calcium increase in coronary artery smooth muscle cells.* Am J Physiol, 1997. **272**(4 Pt 2): p. H1996-2003.
106. Kolodgie, F.D., et al., *Estradiol attenuates directed migration of vascular smooth muscle cells in vitro.* Am J Pathol, 1996. **148**(3): p. 969-76.
107. Bopassa, J.C., et al., *A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury.* Am J Physiol Heart Circ Physiol, 2010. **298**(1): p. H16-23.
108. Broughton, B.R., A.A. Miller, and C.G. Sobey, *Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries.* Am J Physiol Heart Circ Physiol, 2010. **298**(3): p. H1055-61.
109. Deschamps, A.M. and E. Murphy, *Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats.* Am J Physiol Heart Circ Physiol, 2009. **297**(5): p. H1806-13.
110. Ding, Q., et al., *Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30.* Am J Physiol Cell Physiol, 2009. **297**(5): p. C1178-87.
111. Filice, E., et al., *A new membrane G protein-coupled receptor (GPR30) is involved in the cardiac effects of 17beta-estradiol in the male rat.* J Physiol Pharmacol, 2009. **60**(4): p. 3-10.
112. Haas, E., et al., *Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis.* Hypertension, 2007. **49**(6): p. 1358-63.
113. Haas, E., et al., *Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity.* Circ Res, 2009. **104**(3): p. 288-91.
114. Lindsey, S.H., et al., *Chronic treatment with the G protein-coupled receptor 30 agonist G-1 decreases blood pressure in ovariectomized mRen2.Lewis rats.* Endocrinology, 2009. **150**(8): p. 3753-8.
115. Meyer, M.R., E.R. Prossnitz, and M. Barton, *The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function.* Vascul Pharmacol, 2011. **55**(1-3): p. 17-25.
116. Lindsey, S.H., et al., *Vasodilation in response to the GPR30 agonist G-1 is not different from estradiol in the mRen2.Lewis female rat.* J Cardiovasc Pharmacol, 2011. **57**(5): p. 598-603.
117. Meyer, M.R., et al., *Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780.* Pharmacology, 2010. **86**(1): p. 58-64.

118. Meyer, M.R., et al., *Deletion of G protein-coupled estrogen receptor increases endothelial vasoconstriction.* Hypertension, 2012. **59**(2): p. 507-12.
119. Martensson, U.E., et al., *Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice.* Endocrinology, 2009. **150**(2): p. 687-98.
120. Feldman, R.D., et al., *A common hypofunctional genetic variant of GPER is associated with increased blood pressure in women.* Br J Clin Pharmacol, 2014. **78**(6): p. 1441-52.
121. Han, G., et al., *Essential role of the 90-kilodalton heat shock protein in mediating nongenomic estrogen signaling in coronary artery smooth muscle.* J Pharmacol Exp Ther, 2009. **329**(3): p. 850-5.
122. Ushio-Fukai, M., *Vascular signaling through G protein-coupled receptors: new concepts.* Curr Opin Nephrol Hypertens, 2009. **18**(2): p. 153-9.
123. Karas, R.H., et al., *Effects of estrogen on the vascular injury response in estrogen receptor alpha, beta (double) knockout mice.* Circ Res, 2001. **89**(6): p. 534-9.
124. Sharma, G., et al., *GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state.* Endocrinology, 2013. **154**(11): p. 4136-45.
125. Recchia, A.G., et al., *The G protein-coupled receptor 30 is up-regulated by hypoxia-inducible factor-1alpha (HIF-1alpha) in breast cancer cells and cardiomyocytes.* J Biol Chem, 2011. **286**(12): p. 10773-82.
126. Patel, V.H., et al., *G-protein coupled estrogen receptor 1 expression in rat and human heart: Protective role during ischaemic stress.* Int J Mol Med, 2010. **26**(2): p. 193-9.
127. Ullrich, N.D., et al., *Genomic deletion of estrogen receptors ERalpha and ERbeta does not alter estrogen-mediated inhibition of Ca2+ influx and contraction in murine cardiomyocytes.* Am J Physiol Heart Circ Physiol, 2008. **294**(6): p. H2421-7.
128. Delbeck, M., et al., *Impaired left-ventricular cardiac function in male GPR30-deficient mice.* Mol Med Rep, 2011. **4**(1): p. 37-40.
129. Jessup, J.A., et al., *Attenuation of salt-induced cardiac remodeling and diastolic dysfunction by the GPER agonist G-1 in female mRen2.Lewis rats.* PLoS One, 2010. **5**(11): p. e15433.
130. Weil, B.R., et al., *Signaling via GPR30 protects the myocardium from ischemia/reperfusion injury.* Surgery, 2010. **148**(2): p. 436-43.
131. Zhang, B., et al., *Estradiol and G1 reduce infarct size and improve immunosuppression after experimental stroke.* J Immunol, 2010. **184**(8): p. 4087-94.
132. Hsieh, Y.C., et al., *G protein-coupled receptor 30-dependent protein kinase A pathway is critical in nongenomic effects of estrogen in attenuating liver injury after trauma-hemorrhage.* Am J Pathol, 2007. **170**(4): p. 1210-8.
133. Meyer, M.R., et al., *Non-genomic regulation of vascular cell function and growth by estrogen.* Mol Cell Endocrinol, 2009. **308**(1-2): p. 9-16.
134. Abdelhamid, R., et al., *Benzothiophene Selective Estrogen Receptor Modulators Provide Neuroprotection by a novel GPR30-dependent Mechanism.* ACS Chem Neurosci, 2011. **2**(5): p. 256-268.
135. Prossnitz, E.R. and M. Barton, *Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER.* Prostaglandins Other Lipid Mediat, 2009. **89**(3-4): p. 89-97.

136. Hulley, S., et al., *Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group.* JAMA, 1998. **280**(7): p. 605-13.
137. Rossouw, J.E., et al., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial.* JAMA, 2002. **288**(3): p. 321-33.
138. Barrett-Connor, E., et al., *Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women.* N Engl J Med, 2006. **355**(2): p. 125-37.
139. Collins, P., et al., *Effects of the selective estrogen receptor modulator raloxifene on coronary outcomes in the Raloxifene Use for The Heart trial: results of subgroup analyses by age and other factors.* Circulation, 2009. **119**(7): p. 922-30.
140. Cummings, S.R., et al., *Lasofoxifene in postmenopausal women with osteoporosis.* N Engl J Med, 2010. **362**(8): p. 686-96.
141. Ensrud, K., et al., *Lasofoxifene and cardiovascular events in postmenopausal women with osteoporosis: Five-year results from the Postmenopausal Evaluation and Risk Reduction with Lasofoxifene (PEARL) trial.* Circulation, 2010. **122**(17): p. 1716-24.
142. Feng, X.H. and R. Derynck, *Specificity and versatility in tgf-beta signaling through Smads.* Annu Rev Cell Dev Biol, 2005. **21**: p. 659-93.
143. Bobik, A., *Transforming growth factor-betas and vascular disorders.* Arterioscler Thromb Vasc Biol, 2006. **26**(8): p. 1712-20.
144. ten Dijke, P. and H.M. Arthur, *Extracellular control of TGFbeta signalling in vascular development and disease.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 857-69.
145. Heldin, C.H., K. Miyazono, and P. ten Dijke, *TGF-beta signalling from cell membrane to nucleus through SMAD proteins.* Nature, 1997. **390**(6659): p. 465-71.
146. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.
147. Schmierer, B. and C.S. Hill, *TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility.* Nat Rev Mol Cell Biol, 2007. **8**(12): p. 970-82.
148. Oh, S.P., et al., *Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2626-31.
149. Goumans, M.J., et al., *Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors.* EMBO J, 2002. **21**(7): p. 1743-53.
150. David, L., et al., *Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells.* Blood, 2007. **109**(5): p. 1953-61.
151. Goumans, M.J., et al., *Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling.* Mol Cell, 2003. **12**(4): p. 817-28.
152. Deng, G., et al., *Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release?* J Cell Biol, 1996. **134**(6): p. 1563-71.
153. Valdimarsdottir, G., et al., *Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic*

protein-induced activation of endothelial cells. Circulation, 2002. **106**(17): p. 2263-70.
154. Goumans, M.J., Z. Liu, and P. ten Dijke, *TGF-beta signaling in vascular biology and dysfunction.* Cell Res, 2009. **19**(1): p. 116-27.
155. Zhu, H., et al., *A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation.* Nature, 1999. **400**(6745): p. 687-93.
156. Murakami, G., et al., *Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads.* Mol Biol Cell, 2003. **14**(7): p. 2809-17.
157. De Robertis, E.M. and H. Kuroda, *Dorsal-ventral patterning and neural induction in Xenopus embryos.* Annu Rev Cell Dev Biol, 2004. **20**: p. 285-308.
158. ten Dijke, P., M.J. Goumans, and E. Pardali, *Endoglin in angiogenesis and vascular diseases.* Angiogenesis, 2008. **11**(1): p. 79-89.
159. Sankar, S., et al., *Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor beta 2.* J Biol Chem, 1995. **270**(22): p. 13567-72.
160. Kirkbride, K.C., et al., *Bone morphogenetic proteins signal through the transforming growth factor-beta type III receptor.* J Biol Chem, 2008. **283**(12): p. 7628-37.
161. Zhang, Y.E., *Non-Smad pathways in TGF-beta signaling.* Cell Res, 2009. **19**(1): p. 128-39.
162. Moustakas, A. and C.H. Heldin, *Non-Smad TGF-beta signals.* J Cell Sci, 2005. **118**(Pt 16): p. 3573-84.
163. Ding, R., et al., *Endothelial-mesenchymal interactions in vitro reveal molecular mechanisms of smooth muscle/pericyte differentiation.* Stem Cells Dev, 2004. **13**(5): p. 509-20.
164. Seki, T., K.H. Hong, and S.P. Oh, *Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development.* Lab Invest, 2006. **86**(2): p. 116-29.
165. Yao, Y., et al., *Activin-like kinase receptor 1 (ALK1) in atherosclerotic lesions and vascular mesenchymal cells.* Cardiovasc Res, 2007. **74**(2): p. 279-89.
166. Goumans, M.J. and C. Mummery, *Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice.* Int J Dev Biol, 2000. **44**(3): p. 253-65.
167. Arthur, H.M., et al., *Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development.* Dev Biol, 2000. **217**(1): p. 42-53.
168. Chambers, R.C., et al., *Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching.* Am J Pathol, 2003. **162**(2): p. 533-46.
169. Chen, S. and R.J. Lechleider, *Transforming growth factor-beta-induced differentiation of smooth muscle from a neural crest stem cell line.* Circ Res, 2004. **94**(9): p. 1195-202.
170. Lien, S.C., et al., *Phosphatidylinositol 3-kinase/Akt pathway is involved in transforming growth factor-beta1-induced phenotypic modulation of 10T1/2 cells to smooth muscle cells.* Cell Signal, 2006. **18**(8): p. 1270-8.
171. Willette, R.N., et al., *BMP-2 gene expression and effects on human vascular smooth muscle cells.* J Vasc Res, 1999. **36**(2): p. 120-5.

172. Bertolino, P., et al., *Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders.* Chest, 2005. **128**(6 Suppl): p. 585S-590S.
173. Lebrin, F., et al., *Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction.* EMBO J, 2004. **23**(20): p. 4018-28.
174. Mitchell, D., et al., *ALK1-Fc inhibits multiple mediators of angiogenesis and suppresses tumor growth.* Mol Cancer Ther, 2010. **9**(2): p. 379-88.
175. Lamouille, S., et al., *Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis.* Blood, 2002. **100**(13): p. 4495-501.
176. Lechleider, R.J., et al., *Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion.* Dev Biol, 2001. **240**(1): p. 157-67.
177. Chang, H., et al., *Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects.* Development, 1999. **126**(8): p. 1631-42.
178. Lan, Y., et al., *Essential role of endothelial Smad4 in vascular remodeling and integrity.* Mol Cell Biol, 2007. **27**(21): p. 7683-92.
179. Abdalla, S.A. and M. Letarte, *Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease.* J Med Genet, 2006. **43**(2): p. 97-110.
180. Engelman, J.A., J. Luo, and L.C. Cantley, *The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism.* Nat Rev Genet, 2006. **7**(8): p. 606-19.
181. Shiojima, I. and K. Walsh, *Role of Akt signaling in vascular homeostasis and angiogenesis.* Circ Res, 2002. **90**(12): p. 1243-50.
182. Cantley, L.C., *The phosphoinositide 3-kinase pathway.* Science, 2002. **296**(5573): p. 1655-7.
183. Katso, R., et al., *Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer.* Annu Rev Cell Dev Biol, 2001. **17**: p. 615-75.
184. Gerber, H.P., V. Dixit, and N. Ferrara, *Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells.* J Biol Chem, 1998. **273**(21): p. 13313-6.
185. Fujio, Y. and K. Walsh, *Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner.* J Biol Chem, 1999. **274**(23): p. 16349-54.
186. Lawlor, M.A. and D.R. Alessi, *PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?* J Cell Sci, 2001. **114**(Pt 16): p. 2903-10.
187. Thakker, G.D., et al., *The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling.* J Biol Chem, 1999. **274**(15): p. 10002-7.
188. Kimura, H. and H. Esumi, *Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis.* Acta Biochim Pol, 2003. **50**(1): p. 49-59.
189. Zhong, H., et al., *Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics.* Cancer Res, 2000. **60**(6): p. 1541-5.
190. Phung, T.L., et al., *Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin.* Cancer Cell, 2006. **10**(2): p. 159-70.
191. Amano, H., et al., *Adenylate cyclase/protein kinase A signaling pathway enhances angiogenesis through induction of vascular endothelial growth factor in vivo.* Jpn J Pharmacol, 2001. **87**(3): p. 181-8.

192. Cooke, J.P. and D.W. Losordo, *Nitric oxide and angiogenesis.* Circulation, 2002. **105**(18): p. 2133-5.
193. Meili, R., et al., *Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium.* EMBO J, 1999. **18**(8): p. 2092-105.
194. Lee, Y.R., et al., *Up-regulation of PI3K/Akt signaling by 17beta-estradiol through activation of estrogen receptor-alpha, but not estrogen receptor-beta, and stimulates cell growth in breast cancer cells.* Biochem Biophys Res Commun, 2005. **336**(4): p. 1221-6.
195. Ciruelos Gil, E.M., *Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer.* Cancer Treat Rev, 2014. **40**(7): p. 862-71.
196. Guo, R.X., et al., *17 beta-estradiol activates PI3K/Akt signaling pathway by estrogen receptor (ER)-dependent and ER-independent mechanisms in endometrial cancer cells.* J Steroid Biochem Mol Biol, 2006. **99**(1): p. 9-18.
197. Garlich, J.R., et al., *A vascular targeted pan phosphoinositide 3-kinase inhibitor prodrug, SF1126, with antitumor and antiangiogenic activity.* Cancer Res, 2008. **68**(1): p. 206-15.
198. Kong, D., et al., *Antiangiogenic effect of ZSTK474, a novel phosphatidylinositol 3-kinase inhibitor.* Eur J Cancer, 2009. **45**(5): p. 857-65.
199. Liu, C., et al., *PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13.* Acta Biochim Biophys Sin (Shanghai), 2010. **42**(6): p. 396-402.
200. Calabro, P., et al., *Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways.* Circulation, 2004. **110**(21): p. 3335-40.
201. Fernandez-Hernando, C., et al., *Absence of Akt1 reduces vascular smooth muscle cell migration and survival and induces features of plaque vulnerability and cardiac dysfunction during atherosclerosis.* Arterioscler Thromb Vasc Biol, 2009. **29**(12): p. 2033-40.
202. Stabile, E., et al., *Akt controls vascular smooth muscle cell proliferation in vitro and in vivo by delaying G1/S exit.* Circ Res, 2003. **93**(11): p. 1059-65.
203. Luo, Z., et al., *Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity in vivo.* J Clin Invest, 2000. **106**(4): p. 493-9.
204. Scotland, R.S., et al., *Functional reconstitution of endothelial nitric oxide synthase reveals the importance of serine 1179 in endothelium-dependent vasomotion.* Circ Res, 2002. **90**(8): p. 904-10.
205. Ferrara, N., et al., *Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene.* Nature, 1996. **380**(6573): p. 439-42.
206. Carmeliet, P., et al., *Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.* Nature, 1996. **380**(6573): p. 435-9.
207. Carmeliet, P., et al., *Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188.* Nat Med, 1999. **5**(5): p. 495-502.
208. Applanat, M.P., et al., *Vascular endothelial growth factor is a target gene for estrogen receptor and contributes to breast cancer progression.* Adv Exp Med Biol, 2008. **617**: p. 437-44.
209. Heer, K., et al., *Serum vascular endothelial growth factor in breast cancer: its relation with cancer type and estrogen receptor status.* Clin Cancer Res, 2001. **7**(11): p. 3491-4.

210. Liang, Y., R.A. Brekken, and S.M. Hyder, *Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones.* Endocr Relat Cancer, 2006. **13**(3): p. 905-19.
211. Sengupta, K., et al., *Estradiol-induced vascular endothelial growth factor-A expression in breast tumor cells is biphasic and regulated by estrogen receptor-alpha dependent pathway.* Int J Oncol, 2003. **22**(3): p. 609-14.
212. Henricks, L.M., et al., *The use of combinations of monoclonal antibodies in clinical oncology.* Cancer Treat Rev, 2015. **41**(10): p. 859-67.
213. Dimmeler, S., et al., *Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells.* Arterioscler Thromb Vasc Biol, 1999. **19**(3): p. 656-64.
214. Rossig, L., et al., *Nitric oxide inhibits caspase-3 by S-nitrosation in vivo.* J Biol Chem, 1999. **274**(11): p. 6823-6.
215. Dulak, J., et al., *Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells.* Arterioscler Thromb Vasc Biol, 2000. **20**(3): p. 659-66.
216. Ziche, M., et al., *Nitric oxide promotes proliferation and plasminogen activator production by coronary venular endothelium through endogenous bFGF.* Circ Res, 1997. **80**(6): p. 845-52.
217. Ziche, M., et al., *Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P.* J Clin Invest, 1994. **94**(5): p. 2036-44.
218. Murohara, T., et al., *Role of endothelial nitric oxide synthase in endothelial cell migration.* Arterioscler Thromb Vasc Biol, 1999. **19**(5): p. 1156-61.
219. Kashiwagi, S., et al., *NO mediates mural cell recruitment and vessel morphogenesis in murine melanomas and tissue-engineered blood vessels.* J Clin Invest, 2005. **115**(7): p. 1816-27.
220. Ku, D.D., et al., *Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries.* Am J Physiol, 1993. **265**(2 Pt 2): p. H586-92.
221. Yang, R., et al., *Effects of vascular endothelial growth factor on hemodynamics and cardiac performance.* J Cardiovasc Pharmacol, 1996. **27**(6): p. 838-44.
222. van der Zee, R., et al., *Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium.* Circulation, 1997. **95**(4): p. 1030-7.
223. Eagleton, M.J., et al., *Nitric oxide inhibition increases aortic wall matrix metalloproteinase-9 expression.* J Surg Res, 2002. **104**(1): p. 15-21.
224. Stocker, R. and J.F. Keaney, Jr., *Role of oxidative modifications in atherosclerosis.* Physiol Rev, 2004. **84**(4): p. 1381-478.
225. Wang, W., et al., *Inhibition of endogenous nitric oxide in the heart enhances matrix metalloproteinase-2 release.* Br J Pharmacol, 2005. **145**(1): p. 43-9.
226. Sarkar, R., et al., *Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells.* Circ Res, 1996. **78**(2): p. 225-30.
227. von der Leyen, H.E., et al., *Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial cell nitric oxide synthase gene.* Proc Natl Acad Sci U S A, 1995. **92**(4): p. 1137-41.
228. Iafrati, M.D., et al., *Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice.* Nat Med, 1997. **3**(5): p. 545-8.
229. Sullivan, T.R., Jr., et al., *Estrogen inhibits the response-to-injury in a mouse carotid artery model.* J Clin Invest, 1995. **96**(5): p. 2482-8.

230. Karas, R.H., B.L. Patterson, and M.E. Mendelsohn, *Human vascular smooth muscle cells contain functional estrogen receptor.* Circulation, 1994. **89**(5): p. 1943-50.
231. Hogg, M.E., et al., *The role of estrogen receptor alpha and beta in regulating vascular smooth muscle cell proliferation is based on sex.* J Surg Res, 2012. **173**(1): p. e1-10.
232. Rich, T.C. and J.W. Karpen, *Review article: cyclic AMP sensors in living cells: what signals can they actually measure?* Ann Biomed Eng, 2002. **30**(8): p. 1088-99.
233. Aronica, S.M., W.L. Kraus, and B.S. Katzenellenbogen, *Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription.* Proc Natl Acad Sci U S A, 1994. **91**(18): p. 8517-21.
234. Szego, C.M. and J.S. Davis, *Inhibition of estrogen-induced elevation of cyclic 3',5'-adenosine monophosphate in rat uterus. I. By beta-adrenergic receptor-blocking drugs.* Mol Pharmacol, 1969. **5**(5): p. 470-80.
235. Dupont-Mairesse, N., et al., *Mechanism of estrogen action--independence of several responses of the rat uterus from the early increase in adenosine 3',5'-cyclic monophosphate.* J Steroid Biochem, 1974. **5**(2): p. 173-7.
236. Keung, W., et al., *Non-genomic activation of adenylyl cyclase and protein kinase G by 17beta-estradiol in vascular smooth muscle of the rat superior mesenteric artery.* Pharmacol Res, 2011. **64**(5): p. 509-16.
237. Namkoong, S., et al., *Prostaglandin E2 stimulates angiogenesis by activating the nitric oxide/cGMP pathway in human umbilical vein endothelial cells.* Exp Mol Med, 2005. **37**(6): p. 588-600.
238. Hashimoto, A., et al., *Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism.* Atherosclerosis, 2006. **189**(2): p. 350-7.
239. Namkoong, S., et al., *Forskolin increases angiogenesis through the coordinated cross-talk of PKA-dependent VEGF expression and Epac-mediated PI3K/Akt/eNOS signaling.* Cell Signal, 2009. **21**(6): p. 906-15.
240. Song, H., et al., *[Estradiol enhances the proliferation and migration of Ishikawa cells by promotion of angiogenesis induced by activation of NF-kappaB via AKT pathway].* Zhonghua Zhong Liu Za Zhi, 2014. **36**(11): p. 811-5.
241. Zhang, L.D., et al., *Downregulation of ERRalpha inhibits angiogenesis in human umbilical vein endothelial cells through regulating VEGF production and PI3K/Akt/STAT3 signaling pathway.* Eur J Pharmacol, 2015.
242. Kawamoto, A. and T. Asahara, *Role of progenitor endothelial cells in cardiovascular disease and upcoming therapies.* Catheter Cardiovasc Interv, 2007. **70**(4): p. 477-84.
243. Melero-Martin, J.M., et al., *In vivo vasculogenic potential of human blood-derived endothelial progenitor cells.* Blood, 2007. **109**(11): p. 4761-8.
244. Au, P., et al., *Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels.* Blood, 2008. **111**(3): p. 1302-5.
245. Morales, D.E., et al., *Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model.* Circulation, 1995. **91**(3): p. 755-63.
246. Dulak, J., et al., *Heme oxygenase-1 and carbon monoxide in vascular pathobiology: focus on angiogenesis.* Circulation, 2008. **117**(2): p. 231-41.

247. Munaron, L., *Intracellular calcium, endothelial cells and angiogenesis.* Recent Pat Anticancer Drug Discov, 2006. **1**(1): p. 105-19.
248. Han, G., et al., *GPER: a novel target for non-genomic estrogen action in the cardiovascular system.* Pharmacol Res, 2013. **71**: p. 53-60.
249. Holm, A., et al., *The GPER1 agonist G-1 attenuates endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle.* J Vasc Res, 2011. **48**(4): p. 327-35.
250. De Francesco, E.M., et al., *GPER mediates activation of HIF1alpha/VEGF signaling by estrogens.* Cancer Res, 2014. **74**(15): p. 4053-64.
251. Albanito, L., et al., *G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells.* Cancer Res, 2007. **67**(4): p. 1859-66.
252. Gao, F., et al., *GPR30 activation opposes estrogen-dependent uterine growth via inhibition of stromal ERK1/2 and estrogen receptor alpha (ERalpha) phosphorylation signals.* Endocrinology, 2011. **152**(4): p. 1434-47.
253. Thomas, P. and J. Dong, *Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption.* J Steroid Biochem Mol Biol, 2006. **102**(1-5): p. 175-9.
254. Mangmool, S. and H. Kurose, *G(i/o) protein-dependent and -independent actions of Pertussis Toxin (PTX).* Toxins (Basel), 2011. **3**(7): p. 884-99.
255. Lu, C., et al., *Pertussis toxin induces angiogenesis in brain microvascular endothelial cells.* J Neurosci Res, 2008. **86**(12): p. 2624-40.
256. Liu, H., et al., *A novel estrogen receptor GPER mediates proliferation induced by 17beta-estradiol and selective GPER agonist G-1 in estrogen receptor alpha (ERalpha)-negative ovarian cancer cells.* Cell Biol Int, 2014. **38**(5): p. 631-8.
257. Guo, R.X., et al., *[Influence of pertussis toxin on GPER-mediated activation of phosphatidylinositol 3-kinase/protein kinase B signaling induced by 17beta-estradiol in endometrial carcinoma cells].* Zhonghua Fu Chan Ke Za Zhi, 2013. **48**(2): p. 129-33.
258. Luo, H., et al., *GPER-mediated proliferation and estradiol production in breast cancer-associated fibroblasts.* Endocr Relat Cancer, 2014. **21**(2): p. 355-69.
259. Yan, Y., et al., *Role of GPER on proliferation, migration and invasion in ligand-independent manner in human ovarian cancer cell line SKOV3.* Cell Biochem Funct, 2015.
260. Ribatti, D. and E. Crivellato, *"Sprouting angiogenesis", a reappraisal.* Dev Biol, 2012. **372**(2): p. 157-65.
261. Liang, C.C., A.Y. Park, and J.L. Guan, *In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro.* Nat Protoc, 2007. **2**(2): p. 329-33.
262. Ross, S. and C.S. Hill, *How the Smads regulate transcription.* Int J Biochem Cell Biol, 2008. **40**(3): p. 383-408.
263. Heldin, C.H., M. Vanlandewijck, and A. Moustakas, *Regulation of EMT by TGFbeta in cancer.* FEBS Lett, 2012. **586**(14): p. 1959-70.
264. Wu, M.Y. and C.S. Hill, *Tgf-beta superfamily signaling in embryonic development and homeostasis.* Dev Cell, 2009. **16**(3): p. 329-43.
265. David, L., J.J. Feige, and S. Bailly, *Emerging role of bone morphogenetic proteins in angiogenesis.* Cytokine Growth Factor Rev, 2009. **20**(3): p. 203-12.

266. Rider, C.C. and B. Mulloy, *Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists.* Biochem J, 2010. **429**(1): p. 1-12.
267. Scharpfenecker, M., et al., *BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis.* J Cell Sci, 2007. **120**(Pt 6): p. 964-72.
268. Tian, F., et al., *Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro.* Biochem Biophys Res Commun, 2010. **392**(3): p. 283-8.
269. Lyden, D., et al., *Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts.* Nature, 1999. **401**(6754): p. 670-7.
270. Roelen, B.A., M.A. van Rooijen, and C.L. Mummery, *Expression of ALK-1, a type 1 serine/threonine kinase receptor, coincides with sites of vasculogenesis and angiogenesis in early mouse development.* Dev Dyn, 1997. **209**(4): p. 418-30.
271. Matsumoto, Y., et al., *Estrogen and glucocorticoid regulate osteoblast differentiation through the interaction of bone morphogenetic protein-2 and tumor necrosis factor-alpha in C2C12 cells.* Mol Cell Endocrinol, 2010. **325**(1-2): p. 118-27.
272. Matsumoto, Y., et al., *Estrogen facilitates osteoblast differentiation by upregulating bone morphogenetic protein-4 signaling.* Steroids, 2013. **78**(5): p. 513-20.
273. Scharpfenecker, M., et al., *Ionizing radiation shifts the PAI-1/ID-1 balance and activates notch signaling in endothelial cells.* Int J Radiat Oncol Biol Phys, 2009. **73**(2): p. 506-13.
274. Han, C., et al., *SMAD1 deficiency in either endothelial or smooth muscle cells can predispose mice to pulmonary hypertension.* Hypertension, 2013. **61**(5): p. 1044-52.
275. Tuder, R.M. and N.F. Voelkel, *Angiogenesis and pulmonary hypertension: a unique process in a unique disease.* Antioxid Redox Signal, 2002. **4**(5): p. 833-43.
276. Cunningham, N.S., V. Paralkar, and A.H. Reddi, *Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor beta 1 mRNA expression.* Proc Natl Acad Sci U S A, 1992. **89**(24): p. 11740-4.
277. Massague, J., *TGF-beta signal transduction.* Annu Rev Biochem, 1998. **67**: p. 753-91.
278. Xiao, Y.T., L.X. Xiang, and J.Z. Shao, *Bone morphogenetic protein.* Biochem Biophys Res Commun, 2007. **362**(3): p. 550-3.
279. Yao, Y., et al., *Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein.* Circ Res, 2009. **105**(6): p. 575-84.
280. Finkenzeller, G., S. Hager, and G.B. Stark, *Effects of bone morphogenetic protein 2 on human umbilical vein endothelial cells.* Microvasc Res, 2012. **84**(1): p. 81-5.
281. Langenfeld, E.M. and J. Langenfeld, *Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors.* Mol Cancer Res, 2004. **2**(3): p. 141-9.
282. Groppe, J., et al., *Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding.* Mol Cell, 2008. **29**(2): p. 157-68.

283. Upton, P.D., et al., *Functional characterization of bone morphogenetic protein binding sites and Smad1/5 activation in human vascular cells.* Mol Pharmacol, 2008. **73**(2): p. 539-52.
284. Zhu, W., et al., *A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells.* J Biol Chem, 2007. **282**(26): p. 18676-85.
285. Improta-Brears, T., et al., *Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4686-91.
286. Nakhla, A.M., M.S. Khan, and W. Rosner, *Biologically active steroids activate receptor-bound human sex hormone-binding globulin to cause LNCaP cells to accumulate adenosine 3',5'-monophosphate.* J Clin Endocrinol Metab, 1990. **71**(2): p. 398-404.
287. Tesarik, J. and C. Mendoza, *Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential.* J Clin Endocrinol Metab, 1995. **80**(4): p. 1438-43.
288. Benten, W.P., et al., *Estradiol signaling via sequestrable surface receptors.* Endocrinology, 2001. **142**(4): p. 1669-77.
289. Rodriguez, J., M.J. Garcia de Boto, and A. Hidalgo, *Mechanisms involved in the relaxant effect of estrogens on rat aorta strips.* Life Sci, 1996. **58**(7): p. 607-15.
290. Martinez, C., et al., *Mechanisms of diethylstilbestrol-induced relaxation in rat aorta smooth muscle.* Vascul Pharmacol, 2003. **40**(4): p. 197-204.
291. Dubey, R.K., et al., *Cyclic AMP-adenosine pathway inhibits vascular smooth muscle cell growth.* Hypertension, 1996. **28**(5): p. 765-71.
292. Fortunati, N., et al., *Estradiol induction of cAMP in breast cancer cells is mediated by foetal calf serum (FCS) and sex hormone-binding globulin (SHBG).* J Steroid Biochem Mol Biol, 1999. **70**(1-3): p. 73-80.
293. Rosner, W., et al., *Androgen and estrogen signaling at the cell membrane via G-proteins and cyclic adenosine monophosphate.* Steroids, 1999. **64**(1-2): p. 100-6.
294. Balhuizen, A., et al., *Activation of G protein-coupled receptor 30 modulates hormone secretion and counteracts cytokine-induced apoptosis in pancreatic islets of female mice.* Mol Cell Endocrinol, 2010. **320**(1-2): p. 16-24.
295. Lindsey, S.H., L. Liu, and M.C. Chappell, *Vasodilation by GPER in mesenteric arteries involves both endothelial nitric oxide and smooth muscle cAMP signaling.* Steroids, 2014. **81**: p. 99-102.
296. Fukuhara, S., et al., *Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway.* Mol Cell Biol, 2005. **25**(1): p. 136-46.
297. Liu, F., et al., *Role of cAMP-dependent protein kinase A activity in endothelial cell cytoskeleton rearrangement.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(6): p. L1309-17.
298. De Lorenzo, M.S., et al., *'Reduced malignancy as a mechanism for longevity in mice with adenylyl cyclase type 5 disruption'.* Aging Cell, 2014. **13**(1): p. 102-10.
299. Ohta, Y., et al., *Cyclic AMP enhances Smad-mediated BMP signaling through PKA-CREB pathway.* J Bone Miner Metab, 2008. **26**(5): p. 478-84.
300. Ghayor, C., et al., *cAMP enhances BMP2-signaling through PKA and MKP1-dependent mechanisms.* Biochem Biophys Res Commun, 2009. **381**(2): p. 247-52.

301. Katagiri, T., et al., *Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis.* Genes Cells, 2002. **7**(9): p. 949-60.
302. Lopez-Rovira, T., et al., *Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene.* J Biol Chem, 2002. **277**(5): p. 3176-85.
303. Korchynskyi, O. and P. ten Dijke, *Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter.* J Biol Chem, 2002. **277**(7): p. 4883-91.
304. Wrighton, K.H., X. Lin, and X.H. Feng, *Phospho-control of TGF-beta superfamily signaling.* Cell Res, 2009. **19**(1): p. 8-20.
305. Ji, M. and O.M. Andrisani, *High-level activation of cyclic AMP signaling attenuates bone morphogenetic protein 2-induced sympathoadrenal lineage development and promotes melanogenesis in neural crest cultures.* Mol Cell Biol, 2005. **25**(12): p. 5134-45.
306. Zekas, E. and E.R. Prossnitz, *Estrogen-mediated inactivation of FOXO3a by the G protein-coupled estrogen receptor GPER.* BMC Cancer, 2015. **15**: p. 702.
307. Guan, B.Z., et al., *Activation of G Protein Coupled Estrogen Receptor (GPER) Promotes the Migration of Renal Cell Carcinoma via the PI3K/AKT/MMP-9 Signals.* Cell Adh Migr, 2015: p. 0.
308. Zhang, L., et al., *A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A.* Mol Cell Biol, 2004. **24**(5): p. 2169-80.
309. Zavadil, J., et al., *Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6686-91.
310. Davies, M., et al., *Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways.* J Cell Biochem, 2005. **95**(5): p. 918-31.
311. Lee, N.Y., et al., *Endoglin regulates PI3-kinase/Akt trafficking and signaling to alter endothelial capillary stability during angiogenesis.* Mol Biol Cell, 2012. **23**(13): p. 2412-23.
312. Wylie, S., et al., *The matrix metalloproteinase inhibitor batimastat inhibits angiogenesis in liver metastases of B16F1 melanoma cells.* Clin Exp Metastasis, 1999. **17**(2): p. 111-7.
313. Taraboletti, G., et al., *Inhibition of angiogenesis and murine hemangioma growth by batimastat, a synthetic inhibitor of matrix metalloproteinases.* J Natl Cancer Inst, 1995. **87**(4): p. 293-8.
314. Wang, M., et al., *Matrix metalloproteinase 2 activation of transforming growth factor-beta1 (TGF-beta1) and TGF-beta1-type II receptor signaling within the aged arterial wall.* Arterioscler Thromb Vasc Biol, 2006. **26**(7): p. 1503-9.
315. Gerber, H.P., et al., *Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation.* J Biol Chem, 1998. **273**(46): p. 30336-43.
316. Liu, W., W. Su, and T.M. Roberts, *Discovery of estrogen-responsive genes using an improved method which combines subtractive hybridization and PCR.* Nucleic Acids Res, 1998. **26**(15): p. 3616-8.
317. Shifren, J.L., et al., *Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the*

menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab, 1996. **81**(8): p. 3112-8.
318. Greb, R.R., et al., *Vascular endothelial growth factor in primate endometrium is regulated by oestrogen-receptor and progesterone-receptor ligands in vivo.* Hum Reprod, 1997. **12**(6): p. 1280-92.
319. Krasinski, K., et al., *Estradiol accelerates functional endothelial recovery after arterial injury.* Circulation, 1997. **95**(7): p. 1768-72.
320. Meyer, M.R., et al., *G protein-coupled estrogen receptor protects from atherosclerosis.* Sci Rep, 2014. **4**: p. 7564.
321. De Francesco, E.M., et al., *GPER mediates cardiotropic effects in spontaneously hypertensive rat hearts.* PLoS One, 2013. **8**(8): p. e69322.
322. De Francesco, E.M., et al., *HIF-1alpha/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs).* Breast Cancer Res, 2013. **15**(4): p. R64.
323. Schneider-Poetsch, T., et al., *Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin.* Nat Chem Biol, 2010. **6**(3): p. 209-217.
324. Santin, A.D., et al., *Secretion of vascular endothelial growth factor in ovarian cancer.* Eur J Gynaecol Oncol, 1999. **20**(3): p. 177-81.
325. Bjorndahl, M., et al., *Blockage of VEGF-induced angiogenesis by preventing VEGF secretion.* Circ Res, 2004. **94**(11): p. 1443-50.
326. Rosenkilde, M.M., et al., *The minor binding pocket: a major player in 7TM receptor activation.* Trends Pharmacol Sci, 2010. **31**(12): p. 567-74.
327. Luttrell, L.M., et al., *Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes.* Science, 1999. **283**(5402): p. 655-61.
328. DeFea, K.A., et al., *The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex.* Proc Natl Acad Sci U S A, 2000. **97**(20): p. 11086-91.
329. DeFea, K.A., et al., *beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2.* J Cell Biol, 2000. **148**(6): p. 1267-81.
330. Luttrell, L.M., et al., *Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds.* Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2449-54.
331. Ge, L., et al., *A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis.* J Biol Chem, 2003. **278**(36): p. 34418-26.
332. Hunton, D.L., et al., *Beta-arrestin 2-dependent angiotensin II type 1A receptor-mediated pathway of chemotaxis.* Mol Pharmacol, 2005. **67**(4): p. 1229-36.
333. Ahn, S., et al., *Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor.* J Biol Chem, 2004. **279**(34): p. 35518-25.
334. Li, F., et al., *Activation of GPER Induces Differentiation and Inhibition of Coronary Artery Smooth Muscle Cell Proliferation.* PLoS One, 2013. **8**(6): p. e64771.
335. Corcoran, J.J., et al., *Human uterine and placental arteries exhibit tissue-specific acute responses to 17beta-estradiol and estrogen-receptor-specific agonists.* Mol Hum Reprod, 2014. **20**(5): p. 433-41.
336. Lindsey, S.H., et al., *Reduced vasorelaxation to estradiol and G-1 in aged female and adult male rats is associated with GPR30 downregulation.* Am J Physiol Endocrinol Metab, 2013. **305**(1): p. E113-8.

337. Reslan, O.M., et al., *Subtype-specific estrogen receptor-mediated vasodilator activity in the cephalic, thoracic, and abdominal vasculature of female rat.* J Cardiovasc Pharmacol, 2013. **62**(1): p. 26-40.
338. Pare, G., et al., *Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury.* Circ Res, 2002. **90**(10): p. 1087-92.
339. Sivritas, D., et al., *Antiproliferative effect of estrogen in vascular smooth muscle cells is mediated by Kruppel-like factor-4 and manganese superoxide dismutase.* Basic Res Cardiol, 2011. **106**(4): p. 563-75.
340. Yuan, Y. and J. Xu, *Loss-of-function deletion of the steroid receptor coactivator-1 gene in mice reduces estrogen effect on the vascular injury response.* Arterioscler Thromb Vasc Biol, 2007. **27**(7): p. 1521-7.
341. Bjornstrom, L. and M. Sjoberg, *Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes.* Mol Endocrinol, 2005. **19**(4): p. 833-42.
342. Le Romancer, M., et al., *Regulation of estrogen rapid signaling through arginine methylation by PRMT1.* Mol Cell, 2008. **31**(2): p. 212-21.
343. Ueda, K., et al., *Rapid estrogen receptor signaling mediates estrogen-induced inhibition of vascular smooth muscle cell proliferation.* Arterioscler Thromb Vasc Biol, 2013. **33**(8): p. 1837-43.
344. Shan, J., et al., *Leptin-enhanced neointimal hyperplasia is reduced by mTOR and PI3K inhibitors.* Proc Natl Acad Sci U S A, 2008. **105**(48): p. 19006-11.
345. Garat, C.V., et al., *Inhibition of phosphatidylinositol 3-kinase/Akt signaling attenuates hypoxia-induced pulmonary artery remodeling and suppresses CREB depletion in arterial smooth muscle cells.* J Cardiovasc Pharmacol, 2013. **62**(6): p. 539-48.
346. Gilligan, D.M., A.A. Quyyumi, and R.O. Cannon, 3rd, *Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women.* Circulation, 1994. **89**(6): p. 2545-51.
347. Karas, R.H., et al., *Estrogen inhibits the vascular injury response in estrogen receptor beta-deficient female mice.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15133-6.
348. Tang, Y., et al., *Mechanisms of TGF-beta-induced differentiation in human vascular smooth muscle cells.* J Vasc Res, 2011. **48**(6): p. 485-94.
349. Agrotis, A., et al., *Vascular smooth muscle cells express multiple type I receptors for TGF-beta, activin, and bone morphogenetic proteins.* Biochem Biophys Res Commun, 1996. **219**(2): p. 613-8.
350. Bostrom, K., et al., *Bone morphogenetic protein expression in human atherosclerotic lesions.* J Clin Invest, 1993. **91**(4): p. 1800-9.
351. Kretzschmar, M., J. Doody, and J. Massague, *Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1.* Nature, 1997. **389**(6651): p. 618-22.

Curriculum Vitae

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Publications and Presentations

Publication in Preparations

GPER Stimulates Capillary Formation by Human Umbilical Vein Endothelial Cells Via Activation of ALK1/ SMAD 1/5/8 Pathway.
E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey (2016; in preparation).

Crosstalk between PI3K/ Akt/ cAMP and ALK1/ SMAD1/5/8 Pathways Contributes to GPER Induced Capillary Formation by Human Umbilical Vein Endothelial Cells.
E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey (2016; in preparation).

GPER Activation Inhibits Human Arterial SMC Growth: Evidence for an Essential Role of PI3K/ Akt, but not ALK1/ SMAD1/5/8 Pathway.
E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey (2016; in preparation).

Oral Presentations

E. Unterleutner: *Estrogens and Cardiovascular Protective Actions: Role of GPR30.* 4.6.2015, Zurich, Gynecological- Endocrinological Kolloquium, University Hospital Zurich

E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey**:** *Differential Role of AKT-Pathway in Mediating the Growth Effects of GPER in Human Coronary Artery Smooth Muscle Cells and Human Umbilical Vein Endothelial Cell*s. 9.4.2015, Zürich, 14th Day of Clinical Research, Zurich, University of Zurich- Medical Faculty- Center for Clinical Research

E. Unterleutner: *Role of G-Protein Coupled Estrogen Receptor in Mediating the Vasoprotective Actions of Estradiol*. 27.11.2014, Zurich, Gynecological-Endocrinological Kolloquium, University Hospital Zurich, Presentation

Poster Presentations

E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey: *G-protein coupled Estrogen Receptor Stimulates Capillary Formation by Human Umbilical Vein Endothelial Cells via ALK1-SMAD 1/5/8 Pathway Activation.* 16.-19.9.2015, Washington D.C, USA, AHA Council on Hypertension

L. Rigassi, E. Unterleutner, F. Barchiesi, B. Imthurn, R. K. Dubey: *Inhibition of microRNA-221 by Estradiol contributes to its differential effects on Smooth Muscle Cell growth and Endothelial Cell capillary formation.* 16.-19.9.2015, Washington D.C, USA, AHA Council on Hypertension

E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey: *Differential Role of AKT-pathway in Mediating the Growth Effects of GPER in HCASMCs and HUVECs.* 21.8.2015, Zurich, 11th Symposium of the ZIHP Zurich, University of Zurich

L. Rigassi, E. Unterleutner, F. Barchiesi, B. Imthurn, R. K. Dubey: *Role of microRNA-221 in mediating the protective action of Estradiol in vascular cells.* 21.8.2015, Zurich, 11th Symposium of the ZIHP Zurich, University of Zurich

E. Unterleutner, L. Rigassi, F. Barchiesi, B. Imthurn, R. K. Dubey**:** *Differential Role of AKT-Pathway in Mediating the Growth Effects of GPER in Human Coronary Artery Smooth Muscle Cells and Human Umbilical Vein Endothelial Cell*s. 9.4.2015, Zürich, 14th Day of Clinical Research, Zurich, University of Zurich- Medical Faculty- Center for Clinical Research

E. Unterleutner, F. Barchiesi, B. Imthurn, R. K. Dubey: *G-protein Coupled Estrogen Receptor mediates Capillary formation.* 29.8. 2014, Zurich, 10th Symposium of the ZIHP Zurich, University of Zurich, Winner of the Poster Price

E. Unterleutner, F. Barchiesi, B. Imthurn, R. K. Dubey**:** *G-protein Coupled Estrogen Receptor mediates Capillary formation and Induces Vasculogenic pathway ALK1/pSMAD1/5/8.* 12.6. 2014, Zurich, 13th Day of Clinical Research, University of Zurich- Medical Faculty- Center for Clinical Research